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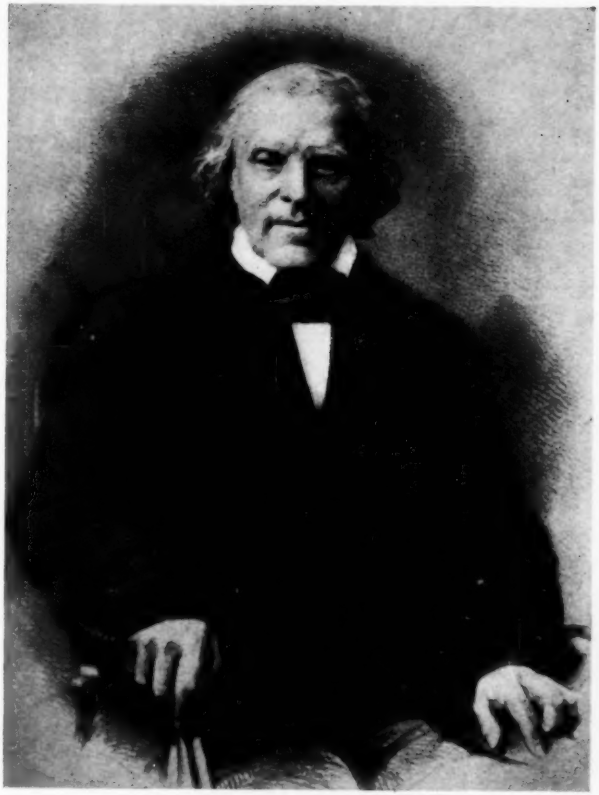

Francois-Vincent Raspail 1794–1878
" The founder of histochemistry "

A REVIEW OF MODERN METHODS IN HISTOCHEMISTRY

BY

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(RECEIVED FOR PUBLICATION JANUARY 2, 1951)

THIS REVIEW is divided into two sections; the first is purely historical, and the second documentary and descriptive in its treatment of modern histochemical methods. Examples of the employment of such methods are given in the second section immediately following the methods to which they refer. The word " modern," in respect of any particular method, refers to usage rather than to origin; nevertheless, a number of the more important methods of which I propose to treat are modern in origin and even ultra-modern in usage.

The History of Histochemistry

It is not easy to write the history of a science which one of its chief exponents (Lison, 1936) believes to have arisen *de novo*, and which many of its supporters consider an invention of the twentieth century. In spite of this we need not doubt that histochemistry began long before 1900, and, taking the subject of the chemistry of the tissues in its broadest sense, we can build up a picture of the journey from its origins in the isolated, unconnected reports of the early nineteenth century down to the comprehensive and expanding science of the present day. The brief tabulated history which appears below is by no means complete; it forms a skeleton on which to hang the otherwise unconnected individual references which follow.

1800-29.—Histochemistry was unknown as a separate science and scarcely practised.

1830-55.—These years saw the beginnings of histochemistry as a science. (Raspail's *Essai de Chimie Microscopique Appliquée à la Physiologie*, 1830, and *Nouveau Système de Chimie Organique*, 1833; Lehmann's *Lehrbuch der physiologischen Chemie*, 1842; Raspail's *New System of Organic Chemistry*, 1834, in translation.)

1856-71.—Histochemistry was mainly biological chemistry, and most of its methods involved tissue destruction. Textbooks on histochemistry began to appear. (*Physiological Chemistry* of Lehmann, 1851, in translation; *Chimie Anatomique* of Robin and Verdeil, 1853; *Chemie der Gewebe des gesammten Thierreichs* of Schlossberger, 1856. *Handbuch der Histologie und Histochemie des Menschen* of Frey, 1867. The first use of aniline dyes in histology was described by Bencke in 1862.)

1872-98.—Part of histochemistry left histology and became attached to physiology, and part remained as biological chemistry. (*Ein Beitrag zur Histochemie*, Miescher, 1873; *Histology and Histochemistry of Man*, Frey, 1874, in translation; *Traité de Chimie Physiologique* of Lehmann, 1883, in translation; and Bunge's *Lehrbuch der physiologischen und pathologischen Chemie*, 1887.)

1899-1929.—The first quarter of the twentieth century saw the rapid expansion of descriptive histopathology. Histologists were concerned with new dyes and staining techniques, and showed less interest in the chemistry of tissue structures. The study of mammalian micro-anatomy was " almost complete " according to Mann. Macallum's *Methoden und Ergebnisse der Mikrochemie*, 1908, and Mann's *Physiological Histology*, 1902, described microchemistry and microphysiology. Other works of this period were the *Review of Recent Developments in Histochemistry*, by Parat, 1927; *Animale Histochemie*, by Patzelt, 1928; *Praktikum der Histochemie*, by Klein, 1929; and *Histochemische methoden*, by Hertwig, 1929.

1930-44.—This stage saw the rebirth of histochemistry and its partial return to the domain of histology. (*Handbook of Chemical Microscopy*, Chamot and Mason, 1930; *Histochemische methoden* of Romeis, 1932; *Die Mikroveraschung als histochemische Hilfsmethode*, by Policard and Okkels, 1932.) The most important work was Lison's *Histochimie Animale* (1936) in which the author proclaimed the new science of histochemistry without tissue destruction.

1945-50.—Since the war there has been considerable expansion of histochemistry and histophysiology by new techniques (Linderström-Lang, *Problems in Histochemistry*, 1936; *Techniques of Histo- and Cyto-chemistry*, Glick, 1949), and the

closing years of this half-century have seen the increasing application of histochemistry to histological and pathological problems.

Baker (1943, 1945) in a fascinating and valuable monograph, and in his book on cytological technique, has drawn attention to the claims of the French microscopist F. V. Raspail to be considered as the founder of histochemistry, and, since the priority and comprehensiveness of these claims are unassailable, his name and portrait head this review as reminders of the ancient origin of the youngest of the pathological sciences. Although earlier references to histochemical practice can be found, such as the use of solutions of iodine for the staining of starch, first described as a reaction by Colin and de Claubry in 1814 and employed in a microscopic study of starch grains by Caventou in 1826, the first clear appreciation of the science of microscopic tissue chemistry came from Raspail (1825a and b; 1829). This author, besides using solutions of iodine as a means of indicating starch in vegetable tissues, produced an aldehyde method for determining protein in the tissues which, modified by many workers since this time, is still applied in histochemistry as the Voisenet-Fürth reaction for tryptophan. Raspail also discovered and applied the xanthoproteic and Liebermann (1887) reactions for protein and tested the reaction of protoplasm with a blue dye obtained from a species of sun spurge, found in the Mediterranean, which turned pink in acid solution. For further information about the astonishing histochemical researches of Raspail, the reader is referred to the original papers, some of which have been translated into English.

Following the initial overtures of Raspail, progress in histochemistry was slow, and very little work which can be described as histochemical, even in the broadest sense, was recorded until after 1860.

Among the oldest histochemical techniques are those for demonstrating iron, and some of these had their origin in the work of Vogel (1845, 1847), who detected iron in the tissues by its conversion to black ferrous sulphide with yellow sulphide of ammonia. In 1867 Perls introduced his Prussian-blue method for demonstrating iron, which remains the method of choice up to the present day. Perls was followed in 1868 by Quinke, who used Vogel's sulphide method. This method also survives to the present day practically unmodified. In 1844 Millon described his reaction for proteins which Hoffmann, in 1853, applied to the characterization of tyrosine. Not until 1888 was the method used in histochemistry, by Leitgeb, although Payen (1843) demonstrated nitrogenous substances in vegetable tissues with mercury proto-nitrate. In 1850, though the result was not published until 1859, Claude Bernard performed his celebrated experiment by the injection into dogs of iron lactate and potassium ferrocyanide, locating the resulting Prussian blue, which developed in the presence of acid, not in the gastric glands but only on the surface of the gastric mucosa. Though *in vivo* techniques are more truly physiological, this particular example may justly be claimed for histochemistry. In 1861 occurred the first reported use of enzymes for tissue digestion which developed, by the end of the century, into the well-recognized technique of enzymal analysis for cytochemical purposes (Kossel and Mathews, 1898). Beale (1861), who was the first exponent of this method, used gastric juice in order to remove unwanted tissues in his study of nerve fibres. As such, the technique was really micro-anatomical. Klebs in 1868 and Struve in 1872 showed that tincture of guaiac gave a blue colour with pus, thus first recording the presence of peroxidases, now well known to occur in the granules of the leucocytes. Brandenburg (1900)

first demonstrated the peroxidase reaction in the latter site, and Ehrlich (1885) performed the " nadi " reaction *in vivo* by injecting alpha naphthol and paraphenylenediamine into animals and observing the formation of indophenol blue. This reaction is now known to denote cytochrome oxidase.

During the next period the work of Schultze (1861), in particular, stimulated histologists to investigate the nature of intracellular tissue. Most of the resulting work cannot be regarded as histochemical. In 1868 Heidenhain showed that ergastoplasm, the deeply basophil substance at the base of secreting gland cells, contained a material which could be precipitated with acetic acid. This is now recognized as ribonucleic acid. In 1870 he described the development of a brown colour in certain structures subjected to treatment with salts of chromium, now known as the chromaffin reaction. The use of enzymes is further exemplified by the work of Miescher (1871), who employed pepsin to free nuclei from cytoplasmic material, and of Stirling (1875), who isolated elastic fibres by means of digestion with gastric juice. These were hardly histochemical techniques in the modern sense, but they serve to illustrate the destructive nature of much of the research into the chemistry of the tissues at that time. In 1873 Miescher isolated nuclear chromatin by making use of its selective affinity for methyl green, and Ehrlich, in 1878–9, observed the effects of heat coagulation in increasing the affinity of haemoglobin for nitro dyes. This last work finds a modern echo in the inquiry into the effects of denaturation on the combination of histochemical reagents with specific groups in the tissues. Cornil (1875) used methyl violet and was the first to note the pink metachromasia given by amyloid when stained with this dye. Flemming (1876) described a cement substance holding together the fibrils which compose the loose connective tissue bundles. This he considered to be of a mucinous nature. A great volume of modern work confirms these observations on the substance now supposed to be the mucopolysaccharide, hyaluronic acid. In 1883 Ehrlich, investigating cases of diabetes, described the use of watery solutions of iodine for the specific staining of glycogen in the tissues. He was not the first to use such a technique, being anticipated by Claude Bernard (1877) in his classical researches.

In the succeeding years a great deal of work was done on the nature of protoplasm, especially by Stöhr (1882), who, as the result of coagulation studies, concluded that a protein substance was present in gastric parietal cells, and by Flemming (1882), Kossel (1882, 1886), Altmann (1886, 1889), Schwarz (1887), and Mann (1890). Altmann, in 1889, developed his method of fixation by freezing and drying, which has become an important modern tool of histochemistry in the hands of Gersh (1932) and his successors. During the last two decades of the nineteenth century histochemistry was submerged in the mass of work arising from the discovery and use of new dyes and the subsequent evolution of new staining techniques. Among the latter, since they have now been realized to have histochemical importance, may be mentioned the anilin-violet method of Gram (1884) and also the myelin methods of Weigert (1884) and Marchi (1892). During this period a great deal of work was being done on the chemical nature of staining, much of which has a modern application in histochemistry. Amongst the works of particular merit are those of Griesbach (1886), who postulated that tissue-dye compounds should have properties differing from those of the free radical; of Unna (1887), who tried to confirm this; and of Lilienfeld (1893), who investigated the staining of mucins. Heine

(1895) is also worthy of note; he observed that segments of chromatin were intensely stained by Millon's reagent and tried, unsuccessfully, to distinguish between nucleoproteins and nucleic acids using mixtures of methyl green and rubin S. In 1895 Macallum demonstrated that, after treatment with sulphuric acid, the nuclei were stainable by the usual methods for iron in the tissues. Since this time, the question of whether this iron is really present in the nuclei, or adsorbed from elsewhere, has been debated at length. The chemical theory of staining was strongly supported by Mathews (1898) in his experimental work with albumins and albumoses, and important researches into the nucleohistones were conducted by Saint-Hilaire (1898) who noticed, in evolving a method for uric acid in the tissues, that nuclei were occasionally stained. He concluded that the presence of histone was responsible for the positive nuclear reaction. Saint-Hilaire also showed that nucleohistones, precipitated in the tissues by acetic acid, were dissociable by means of dilute solutions of hydrochloric acid, leaving the histones *in situ*. Variations on this last theme are widely employed at the present time.

Progress in other methods is represented by the work of Hoyer (1890), who demonstrated metachromasia in the cells of the mucous salivary glands, and by Daddi (1896), who first used Sudan III to stain fat which, when subsequently ingested by animals, was demonstrated in the tissues by its red colour. Sudan IV was proposed as a fat stain by Michaelis in 1901. Before Hoyer's discovery, mucins were stained by techniques making use of their strong basophilia. List (1885) was the first to use Bismarck brown for this purpose, and the same dye, in alcoholic solution, was used by Hardy and Wesbrook (1895) to stain water-soluble mucoproteins and mucopolysaccharides. Enzyme methods are represented by the contributions of Mall (1891), who investigated the swelling of collagen in various solutions, and the action upon it of crude preparations of pepsin and trypsin. Among methods for revealing inorganic salts in the tissues may be mentioned the techniques of Molisch (1893), who stained tissue-iron by converting it to the red thiocyanate, and of Lilienfeld and Monti (1892), who evolved an ammonium molybdate technique for visualizing phosphate, principally in organic form in the nuclei. This method was subsequently modified by Pollacci (1900) and by numerous other workers up to the present time. Finally, though it steps just into the present century and out of history for the purpose of this review, the method for calcium in the tissues which remains in current use was described by von Kóssa in 1901.

When the nineteenth century came to a close, the majority of histologists were occupied in reaping the rich harvest presented by new developments in the art of staining, and few had time to spare for histochemistry. The subject remained for the most part in abeyance until its revival in the 1930s, and its establishment on a modern footing as an independent branch of histology by Lison with his great work *Histochimie Animale*. So much for the past: the present and future of histochemistry remain to be discussed in the succeeding section.

The Practice of Histochemistry

Histochemistry may be defined as the chemistry of the tissues, and cytochemistry as the chemistry of cells. In practice, in order to avoid the use of two words where one will suffice, the word histochemistry can be used to cover the properties of both cellular and extracellular material. If we accept that these properties are both

structural and functional, then histochemistry can be extended to cover matters usually regarded as the province of the physiologists. From the point of view of the histologist and pathologist, however, it is necessary to contract this wider concept and to reintroduce a restricted one. In this restricted sense histochemistry is the investigation of tissue components by applying to microscopical preparations methods of physical and chemical analysis which do not damage the tissues in such a way as to make them unrecognizable. Such methods should be within the compass of all histologists. Only methods which come broadly within this scope are included in the review of the modern methods of histochemistry which follows. The majority can be applied by the histologist to the tissues which he is accustomed to study, subject only to suitable preparation of the material. Few can be described as easy, if easy is taken to mean free of trouble, but good histological staining is itself seldom achieved without considerable effort.

In one or two particular fields the advances achieved by the use of new methods during the past ten years have been spectacular. In others, the use of older methods has continued, often with improved interpretation, as the result of using a number of parallel tests instead of a single one. This principle should always be observed when the nature of any particular material is under investigation. Both the recent advances and some of the older methods will be referred to in this review, and they will be treated broadly under the headings of the various compounds or group of compounds to which they refer. The problems of fixation, and methods for demonstrating enzymes, will be considered in separate sections.

Fixation

No new methods of fixation have been evolved in recent years, but one method in particular, that of freeze-drying, has come into widespread use among histochemists. This method, which was first introduced by Altmann in 1889, depends on the freezing of small pieces of tissue in liquid oxygen, or in isopentane chilled to about $-165°$ C. with liquid oxygen. By this process of rapid freezing all the water in the tissues is converted into ice with the minimum formation of tissue-disrupting ice crystals, which form when material is frozen at higher temperatures. The frozen tissues are then dried *in vacuo* over P_2O_5 for two to five days, being maintained during this period at between -35 and $-70°$ C. The dried tissues, allowed to reach room temperature, are vacuum-embedded in paraffin and cut in the usual manner. It is necessary, before applying most histochemical or ordinary staining techniques to freeze-dried material, to precipitate the proteins in the sections, which are still quite unfixed. For this purpose absolute alcohol is commonly employed.

The method was of little practical use until Gersh (1932) produced the forerunner of all modern freeze-drying apparatus. Since this time its use has been reported by many authors. Scott (1933), Hoerr (1936), Scott and Williams (1936), Packer and Scott (1942), Gersh (1948), and Wang and Grossman (1949) describe the apparatus or the technique for using it, while the application of the method to various problems is discussed by Bensley and Gersh (1933a), Bensley (1933), Bensley and Hoerr (1934), Goodspeed and Uber (1934, 1935), Hoerr and Bensley (1936), Simpson (1941a and b), and others. Its main advantages are that ordinary fixation artifact is avoided; materials in the tissues, including inorganic salts, remain in

those sites which they occupied *in vivo*. Moreover, these materials are not denatured or otherwise altered, and, for instance, the isoelectric point of proteins may still be determined by their minimum solubility in buffers (Catchpole, 1949; Gersh, 1949a). Exception must be made to this statement in respect of many tissue enzymes, which I have found to be reduced in strength by the low temperature involved. This view is contrary to that of Doyle (1950), who found that the peptidase activity of rabbit appendix, using alanylglycine as substrate, was unaffected by freeze-drying and paraffin embedding. The stage has now been reached at which quite a simple apparatus will suffice to give results such as those illustrated in Plate I, Fig. 4, and Figs. 7, 8, and 10. Pancreas, illustrated in Fig. 4, is one of the easiest tissues to freeze-dry, owing to its low water content; other tissues, with a higher water content, are more difficult, and testis is practically impossible.

As far as ordinary fixation is concerned, advances have been made in our knowledge of the fixation of glycogen, ribonucleoprotein, and mucopolysaccharides in particular. Deane, Nesbett, and Hastings (1946) suggested an improvement in the fixation of glycogen which involved the use of ice-cold picro-alcohol-formalin. Lison and Vokaer (1949) recommend for the same purpose either cold alcohol or a mixture of 96% alcohol saturated with picric acid (85 parts), formol (10 parts), and acetic acid (5 parts). This fixative is used at a temperature of $-73°$ C., produced by an acetone-solid CO_2 mixture. Polarization, that is to say, streaming of the glycogen granules to one pole of the cell, still occurs with Lison's method. It can only be avoided by the use of the Altmann-Gersh freeze-drying technique (Figs. 9 and 10). Vallance-Owen (1948) has done considerable service in pointing out that glycogen is quite as adequately fixed by formol-saline as by alcohol or picric-alcohol mixtures. His work was done at room temperatures, however, so that some of the less highly polymerized forms of glycogen, which may be preserved by Lison's refinement, were presumably lost in the fixing fluid. This does not alter the importance of Vallance-Owen's work in showing that glycogen studies can quite well be made on material fixed in formalin. (See methods for glycogen.) The effect of fixatives on enzymal hydrolysis, particularly with ribonuclease, has been studied by Stowell and Zorzoli (1947) and by Tulasne and Vendrely (1947). Both conclude that many fixatives, including plain formalin, give adequate results, but the latter authors obtained the best results with Chabaud's (1942) fixative, which consists of formalin, alcohol, acetic acid, and phenol. Flemming's fluid (Kaufmann, McDonald, and Gay, 1948) and Carnoy (Kaufmann, Gay, and McDonald, 1950) are particularly recommended for the preservation of nucleic acids and proteins, the second fixative leaving them in a condition where they are readily attacked by various hydrolytic enzymes. Many enzymes, hyaluronidases for instance, attack formol-fixed tissues less readily than tissues fixed in non-formalin or alcoholic fixatives. Holmgren and Wilander (1937) first employed 4% basic lead acetate as a fixative for acid mucopolysaccharides, especially for studies on metachromasia. It has since been used for the same purpose by Sylvén (1941), in combination with formalin, but is not worth while for routine use.

Methods for Simple Protein and Amino-acids

Apart from the determination of the iso-electric points of freeze-dried proteins *in situ*, by minimum solubility, numerous workers have attempted to estimate the

iso-electric point of fixed tissue proteins by controlled *p*H staining. Pischinger (1926, 1927) and Zeiger (1930) were the chief exponents of this method. Levine (1940), however, showed that the staining intensity of a protein in buffered dye solutions was dependent on the type of dye and the type of buffer as well as on the concentrations of these and the fixative employed. He rightly stated that the method could not be used for estimation of iso-electric points. It has, nevertheless, proved useful for distinguishing precisely between various substances which exhibit different degrees of basophilia, such as mucopolysaccharides and muco- and nucleo-proteins, and for estimating the degree of tissue basophilia (Dempsey and Singer, 1946; Dempsey, Bunting, Singer, and Wislocki, 1947; Dempsey, Singer, and Wislocki, 1950).

The older microchemical methods for demonstrating protein, e.g., Millon's xanthroproteic method, are still widely employed in histochemistry. They depend on the presence of tyrosine, or of phenolic compounds, and can be largely superseded by modern developments of the diazonium reaction which we owe to Danielli (1947, 1950a). This author, observing that the colour produced by the diazonium reaction for tyrosine, tryptophan, and histidine was too pale to be useful, substituted what I have called his coupled tetrazonium reaction. To the indifferently coloured protein-tetrazonium compound formed in the first part of the reaction, by virtue of the remaining free diazo group, a variety of phenols or amines can be coupled. This process increases the final colour many times, and, since the product is not diffusible, localization is accurate. This method, together with blocking techniques which enable it to be used for distinguishing the three amino-acids separately, renders obsolete the older histochemical methods used for the same purpose.

Workable histochemical modifications of the Sakaguchi reaction for arginine have been evolved by Serra (1944) and by Thomas (1946). The former author's method is theoretically more desirable, and in practice gives a recognizable red with arginine combined in tissue proteins. The specificity of the method is high, but the stability of the colour developed is not. The maximum colour developed during the reaction can seldom be maintained until the stage of examination under the microscope. The method is discussed by Baker (1947), who gives his own modification, and is recommended by Serra (1946) for demonstrating the presence of basic proteins by virtue of their high arginine content.

Three new methods have been evolved for the histochemical demonstration of compounds containing thiols (SH groups) such as cysteine for instance, and these should ultimately replace the deficient nitroprusside and lead acetate methods. They are the thiol-selective coloured organic mercurial method of Bennett (1948) and Bennett and Yphantis (1948), the aldehyde method of Danielli (1950a), and the blocking technique using dinitrofluorobenzene, also developed by Danielli. The last two reactions are as yet imperfectly worked out, according to their originator, but Bennett (1948) describes the successful use of his mercurial reagent for the demonstration of SH groups in biological materials.

Methods for Nucleic Acids and Nucleoproteins

In spite of doubts cast upon its specificity, the classical Feulgen reaction (Feulgen and Rossenbeck, 1924) continues to be employed for the cytochemical localization of thymonucleic (desoxyribonucleic) acid. These doubts have been raised, and sustained, by the work of Choudhuri (1943), Stedman and Stedman (1943a and b,

FIG. 7.—Rabbit kidney. Freeze-dried, paraffin embedded, cut at 6 μ, fixed in 2% osmic acid. Rod-shaped mitochondria in the primary convoluted tubules and their occasional palisade-like arrangement are notable, as is the apparent absence of lumen in many of the tubules. Haemalum and eosin × 550.

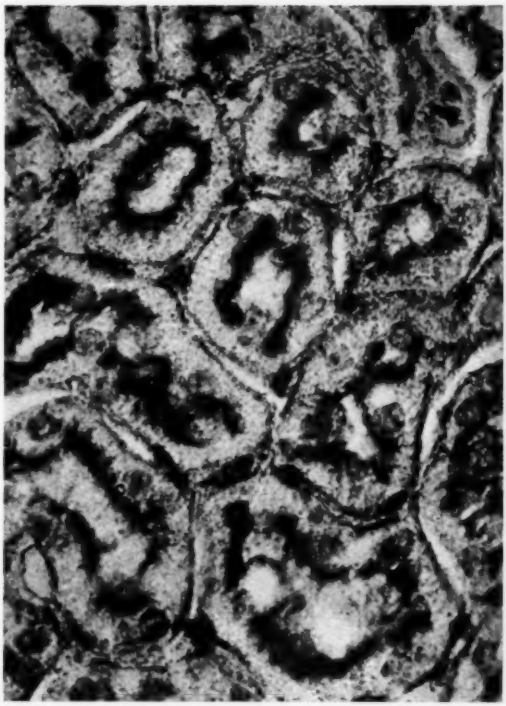

FIG. 8.—Rabbit kidney. Freeze-dried, paraffin embedded, cut at 6 μ, fixed in cold acetone. Three-hour incubation with M/200 phenyl phosphate at *p*H 9.0. To show alkaline phosphatase concentrated in the brush borders of the tubules. Nuclei counterstained with carmalum. × 550.

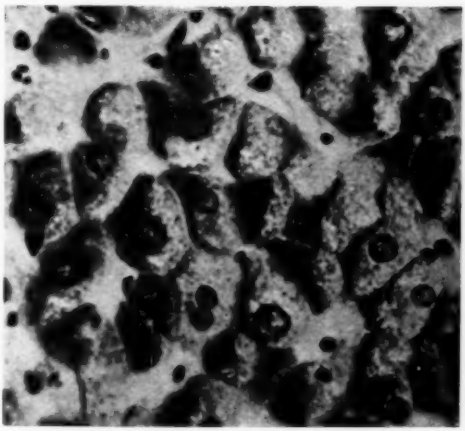

FIG. 9.—Human liver (biopsy specimen). Fixed in alcohol. Polarization of glycogen due to streaming artifact. Best's carmin, haemalum × 460.

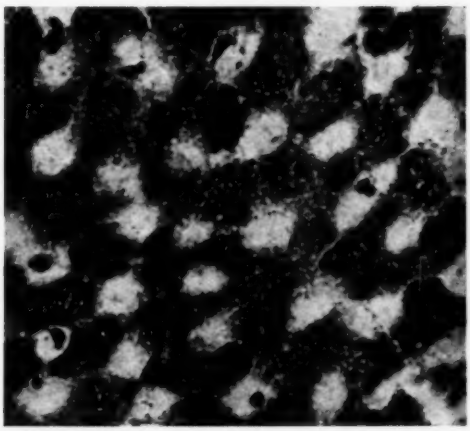

FIG. 10.—Rabbit liver. Freeze-dried, fixed in alcohol. Granules of glycogen are distributed throughout the cytoplasm of the cells. Best's carmine, haemalum × 460.

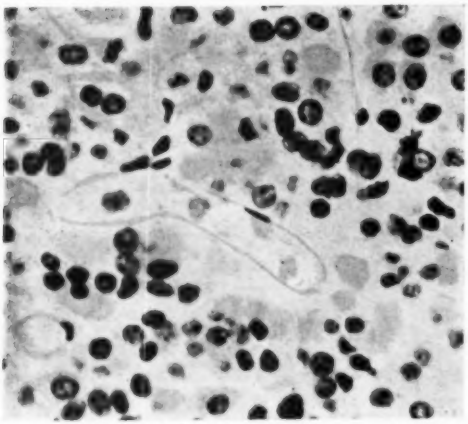

FIG. 11.—Nuclei stained by the Feulgen method for thymonucleic acid (Schiff's reagent after *N* HCl at 60° C. for 8 minutes). Only the chromatin is stained. × 520.

1944), Carr (1945), and Sibatani (1950). These authors believe that the aldehyde-leucofuchsin compound, formed when thymonucleic acid is treated by acid hydrolysis followed by Schiff's reagent, is diffusible, and that it becomes attached to various protein components of the nucleus. On the other hand, a great deal of evidence has been marshalled in support of the specificity of the reaction by Brachet (1946), Dodson (1946), Stacey, Deriaz, Teece, and Wiggins (1946), by Stowell (1946), and, most recently, by Di Stefano (1948), Chong-Fu Li and Stacey (1949), and Overend and Stacey (1949). The latter authors have tried to dispose of the Stedmans' objections by showing that the highly polymerized residue of sperm desoxyribonucleic acid, after the extraction of purines by acid hydrolysis, reacts instantaneously with Schiff's reagent while the supernatant fluid remains colourless for as much as 24 hours. The results obtained by Stedman and Stedman (1950) contradict Stacey's findings. Histochemically the specificity of the Feulgen reaction can be controlled by making use of other reagents for revealing the aldehyde groups produced in the nuclei by mild acid hydrolysis. The results of such a procedure are shown below. Figs. 11 and 12 are of serial sections (though not serial fields), the first stained by the Feulgen reaction (de Tomasi's modification, 1936), and the second subjected to the same acid hydrolysis but treated subsequently with a hydrazide reagent described by Camber (1949) for the localization of ketosteroids (q.v.). This reagent combines with aldehydes as well as ketones, and the resulting compound is revealed by subsequent coupling with tetrazotized dianisidine in alkaline solution, a dark, purplish-blue colour being developed. By this method the localization of chromatin staining in the nuclei is precisely

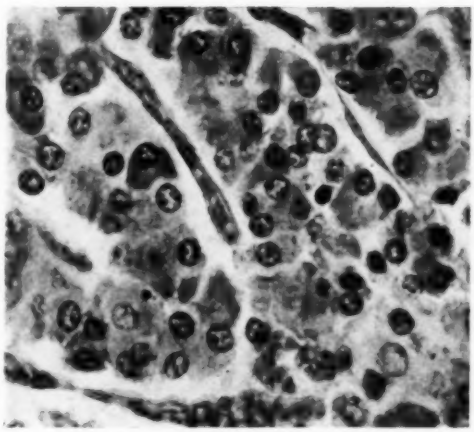

FIG. 12.—Serial section to Fig. 11. Nuclei stained by 2-hydroxy-3-naphthoic acid and diazotized dianisidine after *N* HCl at 60° C. for 8 minutes. Chromatin purple, cytoplasm pink. × 520.

FIG. 14.—Human adrenal gland. Frozen section, 10 μ, mounted in chrome glycerin jelly. Stained by a method reputed to be specific for ketosteroids. A positive reaction is seen in cells of the zona fasciculata. Ashbel-Seligman method × 335.

FIG. 15.—Human skin. Resistance of collagen and reticulin (but not elastin) to the process of benzoylation. Coupled tetrazonium reaction × 235.

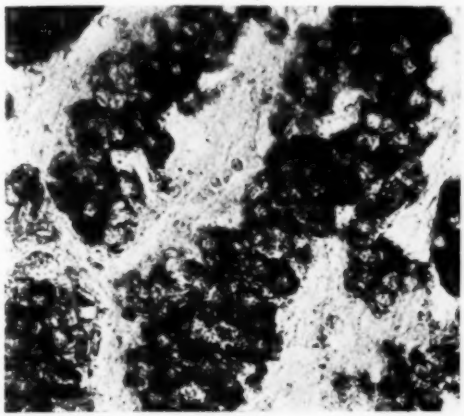

FIG. 14

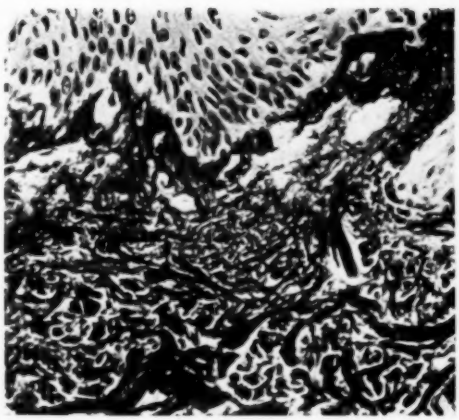

FIG. 15

FIG. 16.—Human tongue. Striated muscle fibres, showing the P.A.S.-positive A bands. Periodic-acid-Schiff, celestin blue-haemalum × 490.

FIG. 17.—Human tongue. Material in the A bands of striated muscle stained after benzoylation. The thinner Z lines were unstained and appear in the photograph because of their refractile properties. Coupled tetrazonium reaction × 1,150.

FIG. 18.—Human anterior hypophysis. Specific staining of the beta granules after the application of moderate heat followed by 18 hours' benzoylation. Coupled tetrazonium reaction × 550.

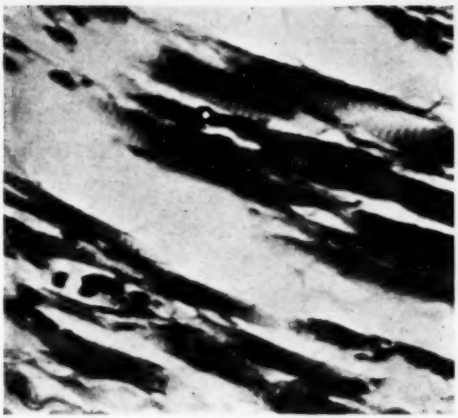

FIG. 16

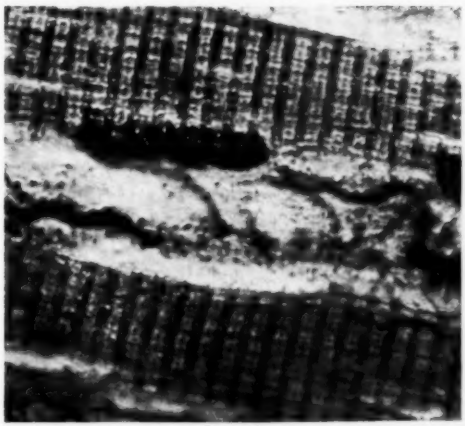

FIG. 17

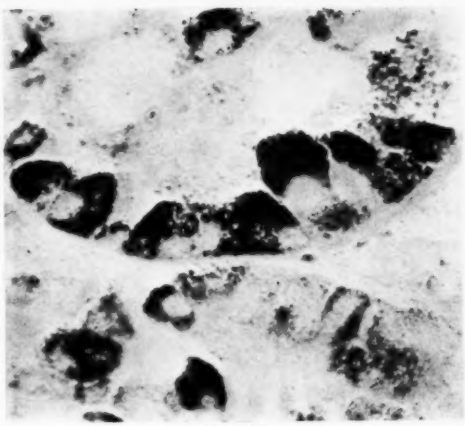

FIG. 18

similar to that shown by the Feulgen reaction. Cytoplasmic detail is visible in Fig. 12 because the hydrazide reagent combines with tissue proteins by virtue of other groups in its molecule and the subsequent treatment imparts a pinkish colour to these protein-hydrazides. A useful modification of the Feulgen technique is described by Rafalko (1946).

The ordinary methods for staining both desoxyribose and ribose nucleic acids depend on the presence of the phosphate ion which confers their characteristic basophilia. Following the example of Brachet (1940, 1942) it is customary to use methyl green-pyronin mixtures for staining and distinguishing between the two types of nucleic acid. After ribonuclease Brachet and other workers have also used toluidine blue, alone or followed by an acid dye, for the purpose of demonstrating both ribonucleic and desoxyribonucleic acids. Material which stains with toluidine blue after ribonuclease is not ribonucleic acid. Both nucleic acids may also be localized by methods making use of their purine and pyrimidine content. Advantage may be taken (Mitchell, 1942; Danielli, 1947) of the fact that purines and pyrimidines react positively in the tetrazonium method already described for tyrosine, tryptophan, and histidine. The reaction with these amino-acids is first prevented by benzoylation or acetylation, after which only the purines and pyrimidines react. Benzoylation is carried out in 10% benzoyl chloride in dry pyridine at room temperature, acetylation with acetic anhydride in dry pyridine at about 100° C. Plasma cell nuclei stained by this method are illustrated in Fig. 13. It is not so useful for cytoplasmic ribonucleotides since these are largely removed by benzoylation. The phosphate ion can also be demonstrated, after its release by nuclease treatment or prolonged acid hydrolysis, by combination *in situ* with ammonium molybdate after the manner of Serra and Queiroz Lopes (1945). These authors employ benzidine in place of the more usual stannous chloride as reducing agent for the phosphomolybdate produced in the first part of the reaction. Of methods demonstrating nucleic acid by virtue of the sugar moiety, Feulgen's reaction for desoxyribose is the best example. There exists also a reaction for both desoxyribose and ribose of nucleic acids described by Turchini, Castel, and Kien (1944), employing phenyl (or methyl) trihydroxy fluorone after acid hydrolysis. This method is not much employed for animal tissues. Variations of the coupled tetrazonium reaction have been used to produce Plate I, Fig. 2, and Figs. 12, 13, 14, and 17. These sections illustrate the scope of the method and some of the problems it may help to answer. They show that many other structures, besides those containing nucleic acids, are

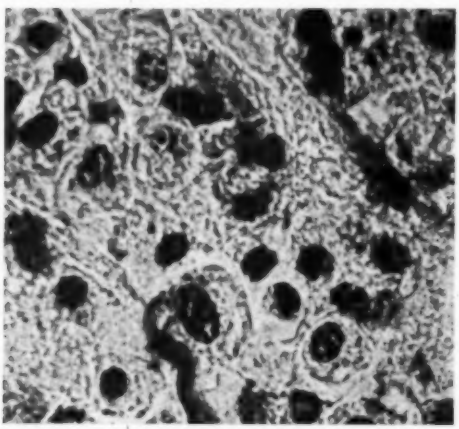

FIG. 13.—A group of plasma cells and, at upper right, three Russell bodies. Simple protein, as well as mucoprotein, Russell bodies stain positively by this technique. Fourteen hours' benzoylation at 22° C. Coupled tetrazonium reaction × 700.

resistant to the effect of benzoylation and acetylation, especially if mild heat has previously been applied to the sections. Collagen and reticulin (Fig. 15), for instance, are invariably resistant. In Plate I, Fig. 1, the mucin of the brush border of the villus stains brilliantly with the periodic-acid-Schiff (P.A.S.) method. Plate I, Fig. 2, shows that it fails to stain by the tetrazonium method after benzoylation. This is probably due to its depolymerization by enzymes in the lumen of the intestine. Fig. 16 shows that the A bands of striated muscle stain strongly by the P.A.S. method, especially in frozen sections. This is only partly due to glycogen. Fig. 17 shows that the A bands stain by the tetrazonium method after benzoylation, as they do after acetylation, whereas the beta granules of the pituitary gland (Fig. 18) withstand benzoylation only (Pearse, 1950b). What cannot be decided on the present evidence is whether these reactions are in each case due to the physical state of the component proteins or whether they are due to the presence of other substances such as adenosine triphosphate (in the A bands) or ribonucleic acid (in the beta granules).

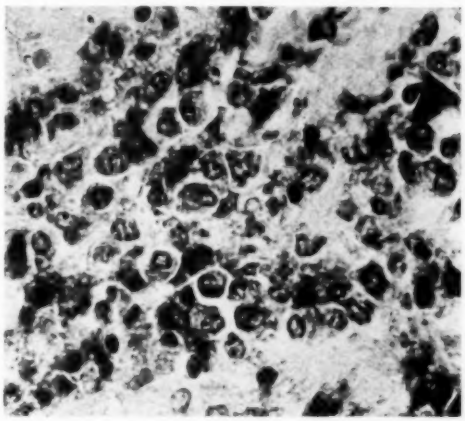

FIG. 19.—A group of plasma cells with granular basophilic cytoplasm containing a large amount of ribonucleic acid. Pyronin-methyl green × 520.

During the past few years a number of extraction techniques for nucleic acids, derived from chemical procedures in the first instance, have been applied to histochemistry.

Sodium Chloride Solutions.—Mirsky and Pollister (1946), for instance, found that ribonucleoprotein and desoxyribonucleoprotein could be extracted from liver by 0.85% and molar NaCl respectively. This action is due to the depolymerization of nucleohistones by salt solutions, especially in molar concentration. White (1950), using bone-marrow smears, has shown that these actions can be demonstrated cytologically by staining in aniline blue-orange G mixtures at *p*H 2 to 3.

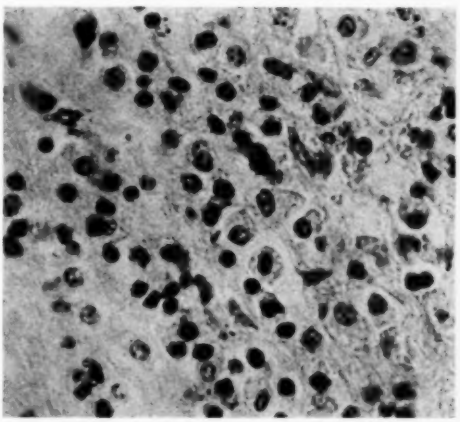

FIG. 20.—A group of plasma cells in a section treated with 10% perchloric acid at 4° C. for 16 hours. Ribonucleic acid has completely disappeared. The nuclei, green after ribonuclease, are red by this method. Pyronin-methyl green × 520.

Perchloric Acid.—After initial work by Ogur and Rosen (1949), Erickson, Sax, and Ogur (1949) evolved a method for the extraction of ribonucleic acid from alcohol-fixed sections, using cold 10% perchloric acid for between four and 18 hours. They also extracted both nucleic acids by treatment with the same reagent at 70° C. for 20 minutes or less. The effect of the perchloric acid hydrolysis was estimated by staining control and treated sections with 1% aqueous toluidine blue. Seshachar and Flick (1949) employed this method to extract ribonucleic acid from protozoan cells, and Sulkin and Kuntz (1950) used it on Zenker-fixed mammalian tissues followed by Mallory's phloxin-methylene blue stain. Erickson and his co-workers and Sulkin and Kuntz both considered that perchloric acid could be substituted for ribonuclease in the histochemical detection of ribonucleic acid. The former authors obtained comparable, and the latter identical, results using ribonuclease on the one hand and cold perchloric acid on the other. This has not been my experience and I consider that their views are premature. Ribonuclease, freed from proteolytic activity, removes ribonucleic acid and nothing else from sections. Cold perchloric acid extraction certainly removes all ribonucleic acid, but also extracts various protein, glycoprotein, and lipoprotein materials. When the optimum times of perchloric acid hydrolysis for tissues fixed in various fixatives are known, as they are for the acid hydrolysis of the Feulgen reaction, for instance, the method will be of considerable value to histologists. In this country, at all events, it should supersede the use of the not easily obtainable ribonuclease. Figs. 19 and 20 illustrate the results of 16 hours' treatment with 10% perchloric acid at 4° C. on a group of plasma cells. Both control and treated sections are stained by the Unna-Pappenheim method.

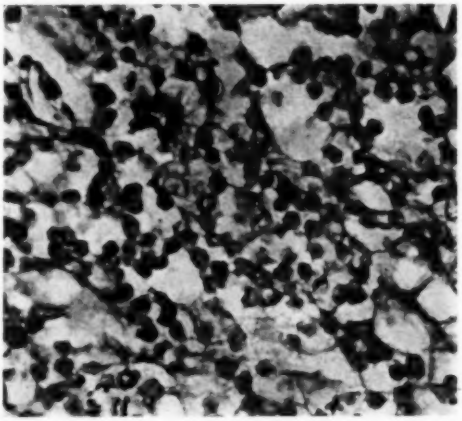

FIG. 21.—A group of plasma cells showing the small affinity of the nuclei for acid dyes. Light green S.F. × 520.

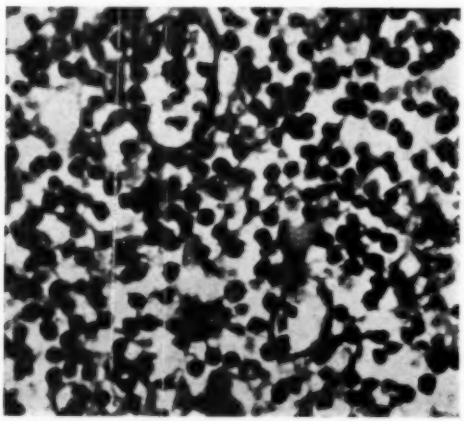

FIG. 22.—Serial section to Fig. 21 treated with 5% trichloroacetic acid at 90° C. for 15 minutes (Schneider extraction). Stained and photographed as Fig. 21. Light green S.F. × 520.

Bile Salts.—Henry and Stacey (1943), in their experiments on the nature of the Gram-positive complex in micro-organisms, employed solutions of bile salts (2% aqueous sodium cholate) at 60° C. in the presence of oxygen for the removal of ribonucleic acids from bacterial cells. This method has been applied by Foster and Wilson (1951) to the extraction of ribonucleic acid from the anterior pituitary beta granules.

Trichloroacetic Acid.—Schneider (1945) used 5% trichloroacetic acid, at 90° C. for 15 minutes, to extract nucleic acids from the tissues. Both desoxy and ribonucleic acids are extracted by this procedure, which has been applied by White (1950) to bone-marrow smears and by Kaufmann, Gay, and McDonald (1950) to onion root tips. Schneider extraction increases the stainability of the nuclei with acid dyes. Figs. 21 and 22 illustrate its application to Helly-fixed tissues followed by staining with light green S.F.

Other Acids.—Other acids, such as hydrochloric, have been employed for nucleoprotein extraction (Sevag, Smolens, and Lackman, 1940; Davidson and Waymouth, 1944), while Caspersson, Hammarsten, and Hammarsten (1935) employed malonic acid for the purpose. Normal HCl at 37° C. for three hours has been employed by Dempsey, Singer, and Wislocki (1950) as a means of reversing the tissue basophilia due to nucleoproteins. Finally, it must not be forgotten that Brachet (1940) showed that ribonucleic acid was removed by incubation in water at 60° C. When an accurate assessment of the optimum time of exposure is available, the perchloric acid method is likely to be the most useful of all the methods in this section to histologists.

Methods for Glycogen and Ascorbic Acid

Recent conceptions of the chemical and physical structure of glycogen have indicated that there are a number of naturally occurring glycogens with different degrees of polymerization and hence with different solubilities (Meyer, 1943). This knowledge has partly explained the difficulties in histological fixation of glycogen, but has made no difference to the demonstration of glycogen in tissue sections. There are still five methods in use for this purpose and each has its adherents. The staining of glycogen with watery solutions of iodine, though certainly the oldest method, is still employed in modern histochemistry (Mancini, 1944) and has its use, especially for freeze-dried material. The original Bauer-Feulgen method described by H. Bauer (1933) employs hydrolysis of the polysaccharide with 4% chromic acid for a short period followed by demonstration of the resulting polyaldehyde with Schiff's solution. It is less often employed in modern histochemistry than the other methods though strongly recommended by C. M. Bensley (1939), and employed by Bunting and White (1950) in their studies on scurvy. Several methods have been devised which employ hydrolysis followed by solutions of silver (Mitchell and Wislocki, 1944; Gomori, 1946c; Arzac, 1947; Arzac and Flores, 1949). Mitchell and Wislocki and Arzac used potassium permanganate as the hydrolysing agent followed by ammoniacal silver solutions, while Gomori employed 10% chromic acid and a hexamine-silver complex for visualizing the aldehyde groups revealed by the hydrolysis. All these methods are similar in effect to Bauer's original method, but reveal mucins and reticulin in addition. Gomori's is probably the best of the three. The periodic-acid-Schiff reaction (McManus, 1946b; Lillie, 1947; Hotchkiss, 1948) reveals glycogen

particularly clearly and has been used for this purpose by Marchese (1947), Wislocki, Rheingold, and Dempsey (1949), Gibb and Stowell (1949), and by McManus and Findley (1949), among others. Lastly, empirical as it is, the ammoniacal carmine solution of Best (1906) cannot be dismissed. It remains in current use by histochemists as well as by histologists (Lison and Vokaer, 1949).

Whichever method is used for demonstrating glycogen in the tissues, control sections incubated with saliva, or with suspensions of malt diastase or ptyalin (Lillie and Greco, 1947), must always be employed if there is any doubt as to the nature of the substance under review. Although the work of Willstätter and Rohdenwald (1934) suggested that glycogen might be bound to protein as desmoglycogen there is no evidence that bound glycogen occurs in any form which resists the action of ptyalin or diastase. That which does so, by whatever method it be revealed, is not glycogen. All the methods in current use for glycogen stain other materials as well, with the exception of Best's stain, properly differentiated. I therefore consider that improved fixation, such as that recommended by Lison, followed by Best's stain and accompanied by diastase-treated control sections, is the most suitable combination for accurate study of the glycogens.

The acid silver technique of Bourne (1933, 1936) for ascorbic acid is mentioned here, as the amount of this substance present in the adrenal gland is often used as a criterion of adrenal function. Barnett, Bourne, and Fisher (1941) modified the reaction to increase its specificity, but the latter is not yet established (K. F. Bauer, 1943; Danielli, 1946). The reaction has been used, particularly on the adrenal gland, by Giroud and Leblond (1936) and by Greep and Deane (1949).

Methods for Mucopolysaccharides, Muco and Glycoproteins

The whole of the modern histochemistry of these substances is bound up with the periodic-acid-Schiff (P.A.S.) reaction of McManus (1946b) and Hotchkiss (1948), which the former author originally described for the staining of mucin. For this purpose, histologically as well as histochemically, it has no peer (Plate I, Fig. 1). It possesses the great advantage of staining neutral as intensely as acid mucopolysaccharides so that gastric mucin, which contains a large amount of the former, stains as strongly as intestinal mucin. The P.A.S. reaction, preferably employing the alcoholic solutions and reducing rinse advocated by Hotchkiss, develops a magenta colour with 1.2 glycols or amino-glycols, which are oxidized to dialdehydes by the periodic acid. Carbonyl groups are oxidized to carboxylic groups and do not, therefore, give a positive reaction. Theoretically, a large number of substances which contain the 1.2 glycol group should give a positive reaction. In practice, if the P.A.S. method is used on paraffin sections after aqueous fixatives, five groups of substances give positive results, and these are not visible in control sections unexposed to periodic acid. They are (1) polysaccharides (glycogen), (2) mucopolysaccharides (mucins, hyaluronic acid, heparin), (3) muco and glycoproteins (mucoids, gonadotrophic, and thyrotrophic hormones, fractions of serum albumin and globulin), (4) glycolipids (kerasin, phrenosin), and (5) phospholipids (lecithin, cephalin, inositol phosphatide, etc.). The presence of reactive substances in the two lipoid groups is determined by staining control frozen and paraffin sections by various methods and, if these suggest that the positive P.A.S. reaction is due to lipoid, by performing the reaction on sections from control blocks fixed in boiling chloroform-methanol for eight to 16

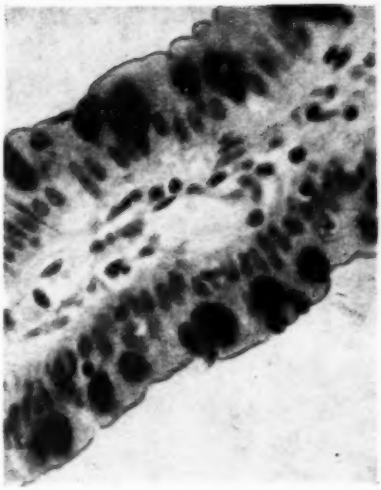

FIG. 1.—Human duodenal villus. Strongly P.A.S.-positive mucin granules are seen in the goblet cells, and less positive mucin in the brush border. Periodic-acid-Schiff, celestin blue-haemalum × 440.

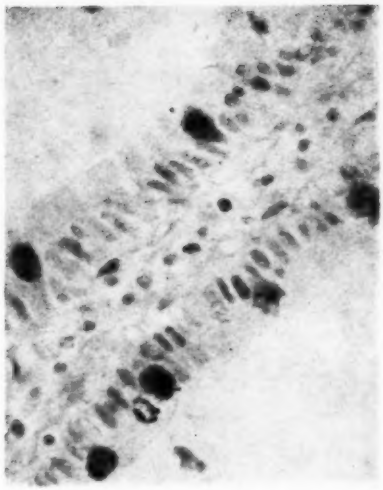

FIG. 2.—A similar villus for comparison with Fig. 1. The granular mucin in the goblet cells stains positively, but the brush border is unstained. Sixteen hours' benzoylation at 22° C. Coupled tetrazonium reaction × 440.

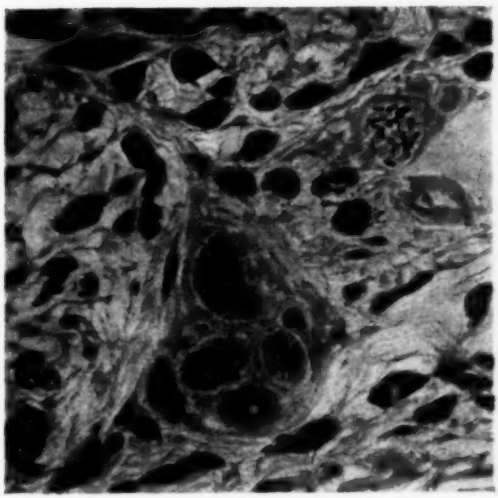

FIG. 3.—Human skin (10 μ frozen section) infiltrated by cells of so-called granular myoblastoma. Sudan black stains the myelin sheaths, fat globules in the sweat glands, and the specific granules of the tumour cells. Sudan black B, carmalum × 150.

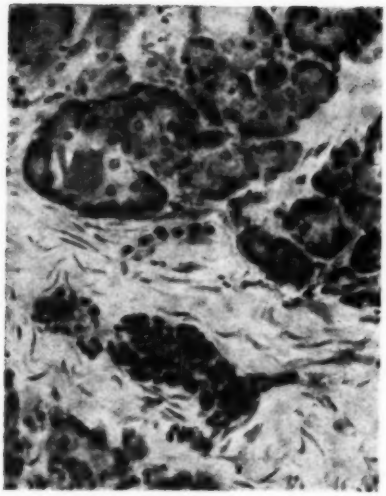

FIG. 4

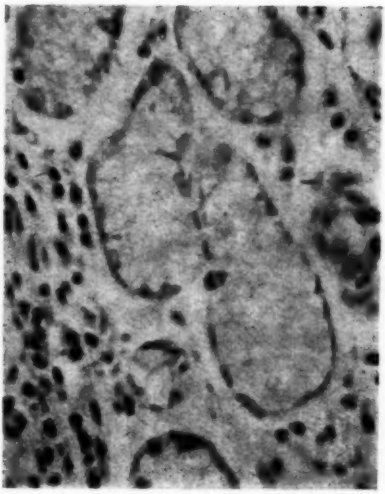

FIG. 5

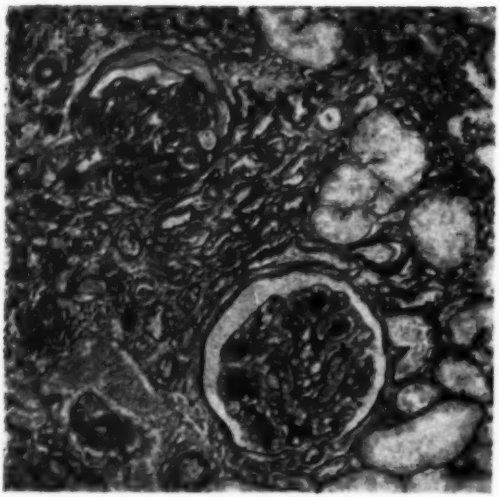

FIG. 6

FIG. 4.—Human pancreas (10 μ freeze-dried section) showing, below, mucopolysaccharide in a duct and, above, mucoprotein secretion in the acini. Periodic-acid-Schiff, toluidine blue × 525.

FIG. 5.—Human stomach. Pyloric glands showing orange-red granules in the argentaffin cells. Diazotized dinitroanisidine (Echtrotsalz B). Iron haematoxylin × 600.

FIG. 6.—Human kidney. Kimmelstiel–Wilson syndrome. Note the hyaline change in afferent arteriole and intraglomerular capillaries. (Section overstained to provide colour contrast.) Periodic-acid-Schiff. Celestin blue-haemalum. Phosphotungstic acid-orange G × 97.

hours. This last procedure will remove all lipoid (with the possible exception of some lipoprotein complexes), and a positive P.A.S. reaction after such a manœuvre is due to substances in the first three groups. Separation of these three groups can also be achieved (see below), making the P.A.S. reaction a useful one in many fields of histochemistry.

Additional methods for the distinction of acid mucopolysaccharides are of three types : (*a*) methods depending on the production of metachromasia, (*b*) methods depending on the degree of basophilia, (*c*) the dialysed iron method (Hale, 1946).

The work of Michaelis and Granick (1945) has introduced the conception that polymer formation is responsible for the development of metachromasia with the thiazine dyes (thionin, toluidine blue, methylene blue). Many dyes in other groups stain metachromatically, pyronin in mast cells, for instance, and celestin blue with mucins (Lendrum, 1947). Lison (1935a) gives a full list. Histochemically, metachromasia can be defined as the staining of a tissue component so that the absorption spectrum of the resulting tissue-dye complex differs sufficiently from that of the original dye and from its ordinary tissue complexes to give a marked contrast in colour. For practical purposes metachromasia refers to the use of dyes of the thiazine group. Polymerization of the substrate, with which the thiazine dye combines, induces polymerization of the dye, and hence metachromasia. New light is thus thrown on the induction of metachromasia which Lison (1936) believed to be a specific histochemical test for higher esters of sulphuric acid (acid mucopolysaccharides), although Feyrter (1936) in the same year had shown that myelin sheaths were strongly metachromatic in frozen sections. Sylvén (1941, 1945) maintained that only such metachromasia as could withstand the process of dehydration with alcohol was the " true " metachromasia due to ester sulphates. One cannot wholly agree with Sylvén's definitions, because alcoholic dehydration is capable of removing all metachromasia if used for long enough, and the amount of his " true " metachromasia in any given section depends on individual technique. Following Michaelis (1947), it is possible to recognize two varieties of metachromasia in histochemistry. This author has shown that toluidine blue has an absorption spectrum with three bands, alpha, beta, and gamma. The monomeric alpha form is blue, the dimeric beta form violet, and the polymeric gamma form is red. Certainly gamma (red), alcohol-resistant metachromasia in paraffin sections is most likely to be due to sulphate esters. Less alcohol-resistant gamma metachromasia, and beta (violet) metachromasia, can be caused by highly polymerized carbohydrate or phosphate-containing compounds. Despite Michaelis's results *in vitro*, nucleic acids occasionally give beta metachromasia in paraffin sections. This feature has been recorded by Wislocki, Bunting, and Dempsey (1947), and I have often observed it. Hempelmann's (1940) claim that distinction between chondroitin and mucoitin sulphuric acids can be made by staining with exceedingly dilute solutions of toluidine blue (which are supposed to stain only the former compounds) cannot be substantiated. Friedenwald (1947) observed that the distinction between meta and orthochromatic staining of tissues could be exaggerated by staining in the presence of high concentrations of magnesium chloride. At *p*H 1.5 nucleic acids fail to stain if the solution is saturated with magnesium chloride, whereas cartilage and mast cells still do so. This technique is a valuable one. The method of Hess and

Hollander (1947) for permanent metachromatic staining of mucin is useful histologically rather than histochemically.

It has long been customary to diagnose the presence of acid mucopolysaccharides by means of their basophilia. More recently, using the method originated by Pischinger (1926, 1927), Dempsey and Singer (1946), Dempsey, Bunting, Singer, and Wislocki (1947), and Dempsey, Singer, and Wislocki (1950) have used a technique for making qualitative estimation of the degree of basophilia registered by tissue compounds. This involves staining with M/2,000 methylene blue at various *p*H levels for 24 hours, mounting sections with minimum dehydration, and applying spectrophotometric measurement to the amount of dye bound by the various tissue components. I have employed this method, without spectrophotometry, using as end-point the virtual extinction of binding capacity for methylene blue (Pearse, 1949, 1950a). The methylene blue extinction method makes possible a comparative estimation of the degree of basophilia manifested by various structures and is particularly useful for distinguishing between acid mucopolysaccharides and mucoproteins.

Hale's (1946) method depends on the combination of dialysed iron, in acid solution, with the sulphate groups of acid mucopolysaccharides or with the uronic acid groups of hyaluronic acid where this occurs in its non-sulphated form. Hale maintains that hyaluronic acid, suitably preserved by alcoholic fixation, is specifically demonstrated by the reaction and that the dialysed iron does not combine with neutral mucopolysaccharides or with proteins. After washing, iron combined in the tissues is visualized by the Prussian-blue reaction. Though the method has apparently been used successfully by Hudack, Blunt, Higbee, and Kearin (1949) and by Ritter and Oleson (1950), who combine it with a peroxidase stain and with the P.A.S. reaction, I have not found that the affinity of fixed tissues for iron is limited to the acid mucopolysaccharides. Hale's reaction is, therefore, not specific, and it is far less easily controlled than methods depending on metachromasia or basophilia. Lillie and Mowry (1949) have investigated the absorption of iron by tissue sections, using M/100 ferric chloride in 1% mannitol, and have shown that mucin, reticulin, and collagen are selectively impregnated.

In modern methods of enzymal analysis at least ten enzymes besides the classical trypsin and pepsin are, or have been, employed. Only three are likely to be required for ordinary use in pathology. These are diastase and ribonuclease, already referred to, and hyaluronidase, recommended by Hale (1946) for the positive identification of hyaluronic acid. There are three common sources of hyaluronidase: (1) streptococcal culture filtrates; (2) clostridial culture filtrates; (3) mammalian testis extracts. Hyaluronidase from all three sources has been used in histochemistry by various workers, but the last source is the most convenient and this hyaluronidase is now obtainable in dried form as a commercial preparation. Such dried testis extracts can be used at about 0.1%, in 0.3% sodium chloride (Bunting and White, 1950) or in 0.1 M-phosphate buffer (Gersh, 1949b), at between *p*H 6.0 and 7.0 and for 12–24 hours at 37° C. Metachromatic material present before and absent after incubation with hyaluronidase is not necessarily hyaluronic acid. Meyer (1947) has shown that testis hyaluronidase can also depolymerize the chondroitin sulphate of cartilage. Control sections, incubated preferably with boiled enzyme solution, must always be employed. Although formalin-fixed material has often been used, alcoholic fixation is preferable.

Methods for Lipoids, Lipoproteins, and Sterols

There are few methods in modern histochemistry primarily designed for the demonstration of lipoids or lipoproteins. Of the older methods a number have fallen into disuse and others continue to be employed although their significance is dubious. For neutral fats the most widely used techniques all depend on the solubility of the inert dis-azo dyes in the fats themselves, and for this purpose Sudan III and Sudan IV are most commonly employed. Kay and Whitehead (1934) found that Sudan III was unsatisfactory as a fat stain and later (1935) proposed and used Sudan IV for this purpose. These same authors (1941) tested a large number of Sudan dyes and concluded that impurities played a part in strengthening the colour imparted to fat in tissue sections. They showed that in mixtures of the Sudan dyes each dissolves independently of the other and the total amount of dye available for staining depends on the number of Sudans present. It is therefore good practice, in a routine fat stain, to use a saturated mixture of Sudan III and Sudan IV in 70% alcohol or in Herxheimer's solution in place of Sudan IV alone. The greatest advance in fat-soluble dye technique has come from the use of Sudan black B, first introduced by Lison (1936) and employed, among other things, for staining the granules in neutrophil leucocytes by Sheehan (1939), McManus (1945), Wislocki and Dempsey (1946), and Eränkö (1950). Sudan black is usually employed as a saturated solution in 70% alcohol, despite the objections of Leach (1938), who recommended 50% diacetin as solvent. This dye has the advantage of being soluble in phospholipids and to some extent in cerebrosides; it can therefore be used for staining them in tissue sections if they are preserved by suitable fixation (Plate I, Fig. 3). McManus (1946a) used a formalin, calcium, cobalt mixture, substituting cobalt for the cadmium of Baker's (1944) fixative for phospholipins. He post-chromed his material when necessary and pointed out that Sudan black might be used on paraffin sections if suitable fixatives were employed. Since formalin alone fixes lipoids to some extent it is always worth while applying the Sudan black technique to formol-fixed paraffin sections when nothing else is available; though a negative result is of no importance, positive results signify formol-fixed lipoid which is usually phosphatide or cerebroside. Other methods in modern use for phosphatides include the Smith-Dietrich method (dichromate-haematoxylin), used by Baud (1948) in a study of perinuclear lipoid layers, the Feyrter (1936) reaction (tartaric acid-thionin on frozen sections) for chromotrope substance, used by Pischinger (1943) and by Pretl (1948), and the acid haematein and pyridine extraction methods described by Baker (1946). Alsterberg (1941) described a new method, said to be specific for phosphatides, involving treatment of unfixed material with "iodine-cyanide" followed by silver chlorate impregnation. At the time of writing I have no experience of this method. Osmic acid is not now employed in histochemistry, and Lorrain-Smith's Nile-blue sulphate, though used as recently as 1950 by Gerebtzoff, Dallemagne, and Philippot, is infrequently employed. Lison (1935a and b) considered that a blue colour with Nile blue was quite unspecific for fatty acids though a red colour signified unsaturated glycerides. More recently Cain (1947), using a new technique, has shown that Nile blue can be used to distinguish neutral lipoids (esters and hydrocarbons) from acidic lipoids (phospholipids).

It has already been stated that the periodic-acid-Schiff reaction is positive with some lipoid materials, notably the cerebrosides and phosphatides. Morrison and

Hack (1950) obtained a brilliant colour in formol-fixed paraffin sections of material from Gaucher's disease stained by this reaction. I found a much less striking development of colour in a single case investigated, but this may have been due to extraction of kerasin by prolonged immersion in formalin. Hack (1949) has found that phosphatides react with P.A.S. *in vitro*; this presumably occurs by virtue of their alpha-amino-alcohol radicles. The P.A.S. method, therefore, can be used in the investigation of formol-fixed lipoid. Preliminary studies indicate that if oxidation is carried out with performic instead of periodic acid, followed by treatment with Schiff's solution (P.F.A.S.), a component which is present in keratin and in the red-cell envelope gives a positive result before any other structures react.

Carleton (1926) and others pointed out that many intracellular lipoidal components were masked. Ciaccio's method of acetone-extraction, and subsequent demonstration by fat-soluble dyes, aimed at revealing these masked lipoids; modern extraction techniques, however, are used for removing rather than revealing. Baker (1946) used pyridine extraction to provide control sections for his method by removing the phospholipid, leaving only certain protein substances to react. Keilig (1944), working on unfixed blocks of human brain, used cold and hot acetone, hot ether, and hot chloroform-methanol to separate the various classes of lipoids. He attached a definite significance to each step, unlike subsequent workers such as Baker and Kent (1950), who used petroleum ether, acetone, alcohol, benzene, and chloroform. As Morrison and Hack found with kerasin, extraction techniques usually fail after formalin fixation and the results are impossible to interpret. Acid haematin and Sudan black are the methods of choice for staining lipoid material other than neutral fat, in conjunction with Keilig types of extraction technique. If Sudan black is used, extraction must not be followed by any fixative or mordant process. Biochemists have not yet decided whether lipoproteins consist of stoichiometrical compounds or of fat micelles covered with protein, or of protein molecules surrounded by layers of lipoid. Whichever they are, they are sometimes resistant to the action of extractives but not usually to hot chloroform-methanol, which removes all lipoids. In tissue structures which contain both protein and formol-fixed lipoid, the two substances are likely to be present in the form of a complex, the lipoid fraction being removable by one or other of Keilig's solvents.

Cholesterol and Ketosteroids.—The following reactions are employed: (1) sudanophilia; (2) birefringence; (3) autofluorescence; (4) Liebermann-Burchardt reaction; (5) Schiff reaction; (6) phenyl- and 2-4 dinitrophenylhydrazine reactions; (7) ammoniacal silver reactions (Reichstein and Shoppee, 1943); (8) hydroxynaphthoic acid hydrazide reactions. All these are subjected to control by using parallel sections treated with cold acetone which removes cholesterol and ketosteroids. Sudanophilia, which has already been discussed, and the silver reactions, which have no advantages for the demonstration of carbonyl groups in the tissues, are not further considered here. The Liebermann-Burchardt reaction, using concentrated sulphuric and acetic acids, produces coloured compounds with unsaturated steroids and is now known to be a general steroid reaction and not specific for cholesterol (Bierry and Gouzon, 1936). It has been used by Dempsey and Bassett (1943) on the ovary, by Everett (1945), and by Greep and Deane (1949) on the adrenal cortex. Both cholesterol and the ketosteroids show birefringence of the

type shown in Fig. 23 (actually a photograph of myelin sheaths by polarized light), and Bennett (1940) increased this birefringence of adrenal cortical lipoids by a modification of the digitonin reaction of Leulier and Revol (1930). Digitonin forms insoluble anisotropic esters with steroids. Steroid compounds also exhibit autofluorescence in ultra-violet light, as shown by Hamperl (1934), Vlès and Ugo (1936), Wadsworth and Crowe (1936), and Deane and Greep (1946). Reactions particularly used for the demonstration of ketosteroids are the two phenylhydrazine reactions, the Schiff reaction, and the naphthoic acid hydrazide reaction. The phenyl hydrazine reaction was employed by Bennett (1939, 1940) and subsequently by Popják (1944) and others, while Dempsey and Wislocki (1946) and later Albert and Leblond (1946) tried to improve the yellow colour of the steroid-phenylhydrazone by using 2-4 dinitrophenylhydrazine. The test with Schiff's reagent is really the plasmal reaction of Feulgen and Voit (1924) which is given especially by the higher fatty aldehydes (acetal phospholipids, plasmalogens). Its application to the adrenal gland in different animal species was reported by Voss (1940). Finally, Camber (1949) and Ashbel and Seligman (1949) independently produced a reaction depending on the formation of coloured hydrazones by combining an arylhydrazide with the tissue steroid and coupling the resulting compound to a diazonium or tetrazonium salt. Using 2-hydroxy 3-naphthoic acid hydrazide and coupling with diazotized dianisidine, the final product has an easily visible bluish-purple colour and is soluble in cold acetone. Fig. 14 illustrates the application of this method to the human adrenal (nuclei unstained; chrome glycerin jelly mountant).

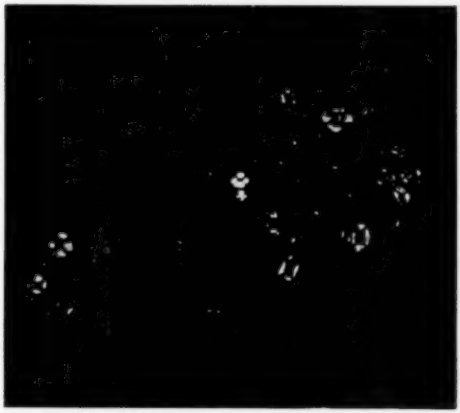

FIG. 23.—Myelin sheaths photographed by polarized light to show maltese-cross birefringence. × 350.

All the methods given above for ketosteroids are non-specific and have been extensively criticized as such. Gomori (1942) considered that the phenylhydrazine reaction signified the presence of plasmalogens in large amounts, and Albert and Leblond (1946) also considered that the phenylhydrazine reaction was equivalent to the plasmal reaction. Boscott and Mandl (1949) stated that all the ketosteroid reactions (they did not review the Ashbel-Seligman method) were non-specific and would be given by an artificial mixture of various lipoid constituents of the tissues. These authors performed amine-aldehyde condensations (Oster and Mulinos, 1944) on sections of human and rat adrenals, and found thereafter that not only was the Schiff reaction for aldehydes negative as expected, but also the dinitrophenylhydrazine reaction. Ketones, therefore, were not present. Aldehydes as well as ketones react by the Ashbel-Seligman method, which can be used in the Feulgen reaction for nucleic acid (Fig. 12) or after periodic acid in place of Schiff's reagent. The specificity of this method, therefore, depends on whether tissue aldehydes react

under the conditions of the experiment. Blocking techniques using semicarbazide (Bennett, 1940) are of no assistance, as they block both aldehydes and ketones from subsequent reaction with hydrazides. Ashbel and Seligman consider that aldehydes do not react by their method, but proof is lacking. I have found that the Schiff and Ashbel-Seligman reactions do not give exactly parallel results on human or rat adrenals, but there is a good deal of overlap between the two, and I consider that the specificity of the latter reaction for ketosteroids remains open to question.

Methods for Alkaline and Acid Phosphatases

Alkaline Phosphatases.—It is quite impossible in a short review to cover all the work on alkaline phosphatase following the original method which was conceived, independently, by Gomori (1939) and by Takamatsu (1939). In the past five years the number of papers referring to the alkaline phosphatases in histochemistry considerably exceeds that of any other single method. This is evidence, at the outset, that the method is reliable and easy, and that it produces a visible and stable result. Too many histochemical techniques proposed in the immediate past have failed to satisfy these criteria. It is necessary, however, to add the warning given by Danielli (1950b) that the Gomori technique is deceptively simple from the point of view of the interpretation of results. Some of the arguments which follow should make this clear. I propose to refer shortly to various papers dealing with the method itself, following this by a short résumé of the points on which its specificity is criticized and by reference to some recent works of general interest to histologists.

Besides the classical method of Gomori, methods using other principles have been evolved; these are briefly reviewed below. Menten, Junge, and Green (1944) produced a histochemical azo dye test, depending on the hydrolysis of calcium beta naphthyl phosphate and the instant reaction *in situ* of the free beta naphthol with diazotized alpha naphthylamine at *p*H 9.4 to give a red colour in sites of alkaline phosphatase activity. Danielli (1946) used phenyl phosphate as well as beta-naphthyl phosphates, coupled with diazotized beta naphthylamine, to accomplish a similar reaction. He also used phenolphthalein phosphate, demonstrating the hydrolysed phenophthalein by exposing the sections to the fumes of ammonia. Manheimer and Seligman (1949) modified the technique of Menten *et al.* by the use of a stable diazotate in place of freshly diazotized alpha naphthylamine. These techniques have proved useful as controls for the specificity of the Gomori method but not for ordinary use. A variation of the original technique (Gomori, 1946a) is commonly used at the present time. It depends on the deposition of calcium phosphate at sites of enzyme activity when sections are incubated with an organic phosphate ester in the presence of calcium ions at *p*H 9, with magnesium ions as activator. The precipitated phosphate is visualized by conversion to the cobalt salt and finally to black cobalt sulphide. Alternatively (Kabat and Furth, 1941) von Kóssa's silver method for calcium may be used. Newman, Feigin, Wolf, and Kabat (1950) give good reasons for objecting to the use of cobalt.

In order that a calcium deposit may take place in the tissues the rate of production of phosphate ions must be high enough for the solubility product of calcium phosphate to be exceeded locally. The establishment of this condition is prevented by many factors: (1) inactivation of the enzyme during fixation and embedding (Emmel, 1946; Danielli, 1946; Stafford and Atkinson, 1948), during the process of mounting

on the slide (Ruyter and Neumann, 1949), and possibly during storage (Lison, 1948); (2) departure from optimum *p*H values (lowering the *p*H below 9.0 interferes by slowing the action of alkaline phosphatase and by increasing the solubility of calcium phosphate); (3) the presence or absence of enzyme activation; (4) the use of unsuitable substrates which are too slowly hydrolysed; the interpretation of results (Gomori, 1950a) is made difficult by (5) false negative reactions due to inactivation or sub-threshold amounts of enzyme; (6) false positive reactions due to other black pigments or to preformed calcium; (7) false localization, due to diffusion of the enzyme during and after fixation (Lison, 1948), or by diffusion of the products of hydrolysis (Jacoby and Martin, 1949; Feigin, Wolf, and Kabat, 1950; Gomori, 1950b). Quantitative estimation is possible according to Gomori (1950b) only by means of laborious comparative experiments. Danielli (1950b), on the other hand, suggests that incubation for a logarithmic series of times gives quantitative appreciation, sites of highest activity appearing before the other sites. It seems that the shorter the incubation can be kept, the more accurate is localization likely to be. In particular the localization of alkaline phosphatase in the nuclei is criticized as being an artifact due to diffusion. Newman, Feigin, Wolf, and Kabat (1950) divided alkaline phosphatases into three groups, one of which is a distinct nuclear phosphatase. Feigin *et al.* (1950) showed that the increased nuclear staining, which occurs adjacent to a locus of intense activity, was due to phosphatase characteristic of that locus and not to the nuclear enzyme. Fig. 8 shows a section of freeze-dried rabbit kidney incubated for three hours with M/200 phenyl phosphate and visualized by the method of von Kóssa. In this short incubation the nuclei were completely unstained and they were counterstained with carmalum for photographic purposes.

Much argument also ranges about the point whether there are a number of different alkaline phosphatases or only one. Evidence derived from *in vitro* studies of resistance to inhibitors and optimum *p*H suggests that a number of phosphatases exist (Belfanti, Contardi, and Ercoli, 1935; Bodansky, 1937; Gould, 1944). Other investigators (Glick, 1946; Dempsey and Deane, 1946; Deane, 1947; Dempsey and Wislocki, 1947; Dempsey, 1949; Friedenwald and Maengwyn-Davies, 1950) believe that various alkaline phosphatases can be distinguished histochemically. Gomori (1950c) demurs. Reis (1937, 1940, 1950) considers that there is a specific 5-nucleotidase acting on adenylic acid at *p*H 7.5 to 8.0, and Gomori (1949a), using adenylic acid at *p*H 9.0, demonstrated this histochemically. Pearse and Reis (unpublished) have also demonstrated 5-nucleotidase histochemically, using adenylic acid at *p*H 7.5. Figs. 24 and 25 illustrate the difference between alkaline phosphatase and 5-nucleotidase in the rat adrenal gland, incubated with either substrate for three hours at 37° C. Such a difference might prove useful for differentiating between cortical and medullary tumours of the adrenal.

The distribution of alkaline phosphatase in normal tissues was described by Gomori (1941a) and by Bourne (1943), and in normal and neoplastic tissues by Kabat and Furth (1941). The kidney has been investigated, especially by Bunting (1948), Soulairac, Desclaux, and Teysseyre (1949a and b), and by McManus and Mowry (1948). The last is a valuable paper dealing with the application of the method to pathological human material. Abolins (1948) and Abolins and Abolins (1949) have investigated the pituitary gland and Fisher and Glick (1947) the skin. Follis (1949) deals with the distribution of alkaline phosphatase in cartilage and bone, and the

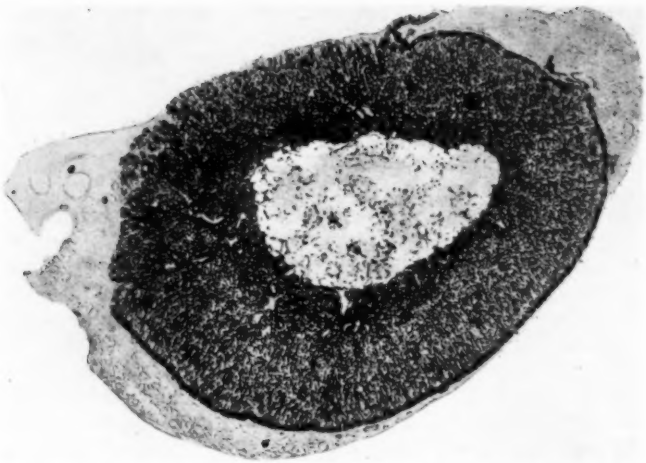

FIG. 24.—Rat adrenal gland incubated for three hours with M/200 phenyl phosphate at *p*H 9.0. Disposition of alkaline phosphatase. × 19.

FIG. 25.—Rat adrenal gland incubated for three hours with M/200 adenylic acid at *p*H 7.5. Disposition of 5-nucleotidase. × 19.

endometrium has been studied by Atkinson and Gusberg (1948) and by Hall (1950). Wachstein and Zak (1946) dealt particularly with the liver. From these and a large number of unquoted studies it appears that the alkaline phosphatase technique can be applied to various pathological materials to give valuable information as to their nature and function.

Acid Phosphatases

Two histochemical techniques exist for the demonstration of acid phosphatases, and neither has achieved the popularity of Gomori's alkaline phosphatase method. The less useful of the two, for histologists, is a variation of the diazo dye technique, described by Seligman and Manheimer (1949), which depends on the hydrolysis of calcium alpha naphthyl phosphate by acid phosphatase at *p*H 5 and coupling of the liberated alpha naphthol with anthraquinone-1-diazonium chloride. The other method, first described by Gomori (1941b), depends, as in the case of alkaline phosphatase, on the liberation of phosphate ions from organic phosphate. In this case the reaction is buffered at *p*H 4.7 to 5.0, at which level calcium phosphate will not precipitate. Lead nitrate is therefore used instead of calcium nitrate in the incubating solution, since its phosphate is insoluble at *p*H 5.0. Treatment with yellow ammonium sulphide converts the precipitated phosphate into the brown sulphide of lead.

Many workers have found Gomori's acid phosphatase technique to be capricious and unreliable (Moog, 1943a; Hard and Lassek, 1946; Lassek, 1947). This last author found it possible to reproduce acid phosphatase pictures in the sciatic nerve of the cat after manœuvres which certainly destroyed all enzymes. He concluded that the acid phosphatase demonstrated histochemically by means of lead salts was a chemical phenomenon. The recent studies of Newman, Kabat, and Wolf (1950) have indicated that non-specific deposition of lead salts is a serious handicap in enzyme localization between *p*H 5.3 and 6.8 and that it occurs occasionally at a lower *p*H. This probably explains Lassek's findings. Gomori (1950c) has given details of an improved acid phosphatase method. He stresses the importance of the ratio of buffer to substrate in improving the constancy of results. It appears that the acid phosphatase technique is a valuable tool in the investigation of pathological conditions provided that two control sections are invariably employed, one inactivated with M/100 sodium fluoride and the other incubated in a medium containing no substrate.

Methods for Other Enzymes

A few years ago perhaps three or four enzymes could be demonstrated by histochemical techniques in animal tissues. Excluding the phosphatases, there are now techniques for at least ten. Brief mention of these is all that space allows. Gomori (1948b) described the demonstration of sites of *phosphamidase* activity, incubating sections at *p*H 5.6 with p-chloranilidophosphonic acid and lead nitrate. The specific enzyme occurred in small amounts in normal tissue, but in large amounts in grey matter and in malignant epithelial tumours. Newman *et al.* (1950) considered that part or all of the staining achieved with this method might be due to non-specific deposition of lead. For *peroxidases* a large number of methods exist employing either benzidine, which is oxidized to a blue compound, or alpha naphthol. A recent application of the alpha naphthol method to blocks of tissue, as opposed to blood smears, is described by Ritter and Oleson (1947, 1950). Keilin (1933) showed that the "nadi" reaction, producing indophenol blue from alpha naphthol and p-aminodimethylaniline, revealed the presence of *cytochrome oxidase-cytochrome C*, and Lison (1936) observed that the reaction should be carried out on fresh tissues in alkaline solution. Moog (1943b) used the reaction to demonstrate cytochrome oxidase in chick embryos. A method for *esterases* has been evolved by Nachlas and Seligman (1949) using the diazo dye principle with beta naphthyl acetate and

diazotized alpha naphthylamine. Gomori (1950b) observes that staining of anomalous sites results with this method, due either to absorption of free naphthol or of the azo dye formed by enzyme action elsewhere. He suggests that more accurate localization can be achieved with alpha naphthyl acetate. Methods for *lipases* were described by Gomori (1945, 1946b), and elaborated later (1949b). Wachstein (1946) modified Gomori's original method, in which sections are incubated with water-soluble unsaturated or saturated fatty acid esters of polyglycols, in the presence of calcium ions. Insoluble calcium soaps are precipitated at the sites of enzyme activity and visualized by conversion to lead salts and, finally, to lead sulphide. Sneath (1950) considers that the lipase technique is not yet sufficiently reliable for use in diagnostic pathology.

Two independent methods exist for the localization of *choline esterase*. In the first (Gomori, 1948a) the choline ester of a higher fatty acid (myristoyl choline) is hydrolysed by the enzyme in acetone-fixed sections at *p*H 8. The resulting free fatty acid is precipitated as its cobalt salt and visualized as sulphide. The authors of the second method (Koelle and Friedenwald, 1949) believed that Gomori's method revealed only non-specific esterases. They incubated fresh frozen sections in a medium containing acetylthiocholine and copper glycinate at *p*H 8. The reaction product, copper thiocholine, was then converted to brown copper sulphide. Seligman, Nachlas, and Cohen (1950) produced a reaction for *sulphatases* using potassium-6-benzoyl-2-naphthyl sulphate as substrate, and coupling the free naphthyl compound to a diazonium salt *in situ*. These same authors described a method for *beta-glucuronidase*, using a similar principle, with 6-bromo-2-naphthol glucuronide as substrate. Friedenwald and Becker (1948) used two methods for this enzyme. The first involves the precipitation of the insoluble red hydroxyphenylazo-beta naphthol from its soluble glucuronide by the action of the enzyme in fresh tissue sections. Localization is good and the method comparatively easy; unfortunately, since the red beta naphthol compound is soluble in alcohol and xylol, only watery mounting media can be used. The second method depends on the hydrolysis of 8-hydroxy-quinoline glycuronide, precipitation of the free quinoline derivative, and its conversion to the ferric salt which is subsequently visualized by the Prussian-blue reaction. The second method was modified by Campbell (1949) for a study of normal and neoplastic tissues; anaplastic carcinomas of animal origin were particularly rich in the enzyme. The method for *amine oxidase* introduced by Oster and Schlossman (1942) was based on the oxidation of tyramine to an aldehyde and its visualization by Schiff's reagent. It has been shown by Gomori (1950a) to be quite unspecific. The *dopa oxidase* technique first described by Bloch (1917) remains in current use, the modification introduced by Laidlaw and Blackberg (1932) being usually employed. Finally, methods exist for demonstrating *dehydrogenases* in fresh plant and animal tissues. These employ triphenyltetrazolium chloride, or its iodo derivatives, which are reduced to insoluble red formazans (Pratt and Dufrenoy, 1948; Black, Kleiner, and Speer, 1950; Atkinson, Melvin, and Fox, 1950). Rutenberg, Gofstein, and Seligman (1950) have prepared a di-tetrazolium salt which is reduced to a blue formazan, more easily localized than the red compound.

Few of these enzyme methods are ready for general application to pathological problems at the present time, with the exception of those for alkaline phosphatase. No doubt the study of glucuronidase in pathological tissues would yield valuable

results. But the more specific of the two methods for this enzyme, using hydroxyquinoline glucuronide, is not simple to perform, while with the other method localization is less reliable and permanent preparations cannot be obtained. The dopa oxidase method is reliable, but its application is limited, while the other methods mentioned have not yet reached the stage at which they can reasonably be employed in pathological investigations.

Miscellaneous Methods

A miscellany of methods of interest to histologists remains to be briefly considered.

Inorganic Anions.—Gersh (1938) described two silver nitrate reagents for visualizing *chloride* on the one hand and chloride, *phosphate*, and *carbonate* on the other in freeze-dried material mounted on slides without contact with water. For this purpose floating out on warm mercury, first suggested by Danielli and used by Harris, Sloane, and King (1950), is particularly suitable.

Inorganic Cations.—The Prussian and Turnbull blue and other methods for iron are critically reviewed by Lillie (1948) and by Bunting (1949), who concludes that the optimum strength of the ferro-cyanide-HCl mixture for the former method is 2% of each, applied for one hour at room temperature. The ammonium sulphide-dinitroresorcinol method of Humphrey (1935) has the two advantages of permanency and freedom from iron-containing reagents. It is far less sensitive than the Prussian-blue test and will not, therefore, be used in critical work. I have not found the 8-hydroxyquinoline method of Thomas and Lavalloy (1935) to be either convenient or reliable. No advances in the technique for demonstration of *calcium* have been made since Cameron's (1930) review of the methods then available. The method of choice remains that of von Kossa, using control sections freed from calcium by immersion in 0.1 M–citrate buffer at *p*H 4.5 for 10 to 20 minutes. Few other anions or cations are likely to require investigation by the pathologist, although methods exist for gold, silver, bismuth, mercury, palladium, and even less probable elements. The sensitivity and specificity of most methods for inorganic substances leave much to be desired, and any advance in the localization of cations as well as anions seems likely to be made by the use of radioactive isotopes and autoradiography, though the latter has grave drawbacks for high-power work.

Various techniques exist for the autoradiographic detection of organically bound radioactive iodine (I^{131}), and they can be carried out by histologists without special difficulty (Bélanger and Leblond, 1946; Evans, 1947; Pelc, 1947; Doniach and Pelc, 1949, 1950). These techniques differ in the manner in which the photographic film or emulsion is applied to the surface of the section containing the radioactive substance. Ordinary histological fixatives are employed and paraffin sections, but contact with water is to be avoided if soluble substances are being studied. Holt, Cowing, and Warren (1949) found considerable loss of radiophosphorus from sections floated out on water. Freeze-dried sections floated out on warm mercury should be ideal for autoradiography, but these authors obtained their best results with alcohol-formalin fixation, dioxane dehydration and clearing, and paraffin embedding.

Two other physical methods, of simple application, are available to the histologist. The polarizing microscope, and the simpler polaroid lenses, remain extremely

useful for determining the presence of doubly refractile fats, though they cannot give clear information as to the nature of these. Cholesterol esters, phosphatides, cerebrosides, steroids, and formol-fixed neutral fat may all be birefringent. Lison (1936) distinguished between simple illumination by polarized light, indicating fat in a crystalline state, and maltese-cross formation, indicating cholesterides or lipins (phosphatides and cerebrosides). It is as well to remember that other structures such as collagen and keratin (Fig. 26) may also be birefringent. Figs. 23 and 24 illustrate the difference between maltese-cross and simple illumination by polarized light. Microscopy using filtered ultra-violet light, from a source such as that suggested by Lempert (1944), has many applications, but few can be considered useful. Natural fluorescence is exhibited by a number of materials in vertebrate tissues when these are exposed to ultra-violet light. The *porphyrins* may be located by their red fluorescence (Grafflin, 1942; Gillman, Gillman, and Brenner, 1945), the *granules of the argentaffin cells* by their golden yellow (Jacobson, 1939), *vitamin A* by its fading green (Popper, 1944), and *riboflavin* by its greenish-yellow (Ellinger, 1938). If we except the demonstration of acid-fast bacilli by means of auramine, the staining of tissues with fluorescent dyes and examination by ultra-violet light has nothing to recommend it. It has been used principally by Popper (1944), using phosphine R, for fats and by Szanto and Popper (1948) for ribonucleic acid, making use of the latter's property of quenching the fluorescence of cytoplasm stained with coriophosphin. Ultra-violet fluorescence is a pretty way of demonstrating argentaffin cell granules, but they are far better shown by using a silver method, such as Masson's (1925) Fontana stain, or by coupling with diazonium salts. Fig. 5 shows argentaffin granules stained by the latter method (Cordier and Lison, 1930; Clara, 1935), using the stable diazotate of nitroanisidine (*Echtrotsalz B*). Gomori (1948c) employed a number of histochemical methods to determine the nature of these granules and concluded that they contained a derivative of resorcinol. In a case of argentaffin carcinoma of the stomach (unpublished) I found that the specific granules in the tumour cells would not reduce Fontana's silver solution in 24 hours and they were detectable only by the diazo method.

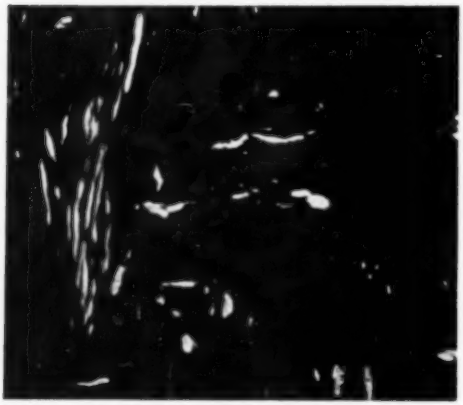

FIG. 26.—Keratin fragments in an epidermoid cyst to show plain birefringence (prisms half rotated). × 350.

In recent years the chemistry of the *melanins* has been further elucidated, but, so far, their cytochemistry remains unaltered. Their property of reducing silver salts is the only one made use of by histologists and histochemists alike. Mason (1948a) considered melanin to be a polymerized indole derivative, and Lea (1949) noted that Ehrlich's dimethylaminobenzaldehyde reaction was positive for indole and melanogen but negative for fully formed melanin. According to Mason

TABLE

RECOMMENDED HISTOCHEMICAL METHODS FOR PARTICULAR SUBSTANCES

Substance	Method	Fixation	Frozen or Paraffin	References
Glycogen	Best	Cold alcoholic or various	P	Lison and Vokaer (1949)
Ascorbic acid	Acid silver	Fresh	F	Bourne (1936)
Protein (tyrosine) ..	Millon	Various	P	Bensley and Gersh (1933b)
Protein (tyrosine) ..	Tetrazonium	Various	P	Danielli (1947)
Arginine	Sakaguchi	Acetic-alcohol-formalin	F or P	Serra (1944)
Thymonucleic acid ..	Feulgen	Carnoy or various	P	Bauer (1932); de Tomasi (1936)
Ribonucleic acid ..	Ribonuclease, pyronin	Carnoy or mercurial	P	Brachet (1940, 1942)
Ribonucleic acid ..	Perchloric acid extraction	Various	P	Erickson, Sax, and Ogur (1949)
Purines : Pyrimidines	Benzoylation and tetrazonium	Various	P	Danielli (1947)
Mucins	P.A.S.	Various	P	Hotchkiss (1948); McManus (1946b)
Mucoprotein	P.A.S.	Preferably formalin-containing	F or P	Hotchkiss (1948)
Mucopolysaccharides ..	Metachromasia	Various	F or P	Lison (1936)
M.P.S. including hyaluronic acid	Metachromasia	Basic lead acetate	P	Holmgren and Wilander (1937)
Hyaluronic acid ..	Dialysed iron hyaluronidase	Carnoy	P	Hale (1946)
Hyaluronic acid ..	Metachromasia hyaluronidase	Formalin or jelly	P	Penney and Balfour (1949)
Mucopolysaccharides ..	Basophilia (quantitative)	Formol-mercury	P	Dempsey and Singer (1946)
Neutral fats	Sudan III and IV	Formalin	F	Kay and Whitehead (1941)
Phospholipid	Sudan black B	Formol-calcium	P	McManus (1946a)
Phospholipid	Acid haematin	Formol-calcium	P	Baker (1947)
Phospholipid	Pyridine extraction	Weak Bouin	P	Baker (1947)
Glycolipid (cerebroside)	P.A.S.	Formalin	P	Morrison and Hack (1950)
Lipoids	Selective extraction	Fresh (fixed by extractive)	P	Keilig (1944)
Cholesterol	Liebermann-Burchardt	Formalin	F	Schultz (1925a, 1925b)
Ketosteroids	Naphthoic acid hydrazide	Formalin	F	Ashbel and Seligman (1949)
Alkaline phosphatase ..	Calcium-cobalt	Cold acetone	P	Gomori (1946a)
Alkaline phosphatase ..	Calcium-silver	Cold acetone	P	Kabat and Furth (1941)
Acid phosphatase ..	Calcium-lead	Cold acetone	P	Gomori (1950c)
Peroxidase	Block technique	Formol-alcohol	P	Ritter and Oleson (1947)
Glucuronidase ..	Hydroxyquinoline	Fresh	F	Friedenwald and Becker (1948)
Dopa oxidase	Dopa	Fresh	F	Laidlaw and Blackberg (1932)
Iron	Prussian blue	Neutral formalin or various	P	Bunting (1949)
I^{131}	Autoradiograph	Alcohol	P	Doniach and Pelc (1949a)
Argentaffin granules ..	Diazo	Formalin-containing	P	Jacobson (1939)
Argentaffin granules ..	Hexamine-silver	Formalin-containing	P	Gomori (1948c)

(1948b) melanin is soluble in alcohol, pyridine, or ethylene chlorohydrin after separation from its protein component. Taft (1949), however, used all these solvents at 22 and 66° C. for up to 24 hours on fixed paraffin sections and unfixed frozen sections without observing any diminution in the amount of melanin present. Oxidation remains the only satisfactory method for removing the colour of melanin, either for diagnostic purposes or where it interferes with the interpretation of another reaction, that for phosphatases, for instance. Three methods are in current use; 1/2,000 permanganate for 24 hours, potassium chlorate in acid alcohol for 15 minutes, and 10 vol. hydrogen peroxide for 24 hours. The last is the most reliable. Of the remaining pigments, only *bilirubin* is of sufficient interest to be noted here. Of the positive histochemical tests for this substance, Gmelin's nitric acid reaction is too violent and Stein's (1935) iodine test neither reliable nor specific. Negative tests, such as the failure to react with ferrocyanide-HCl or ammoniacal silver, and the stability of the pigment to hydrogen peroxide, coupled with staining by means of methylene blue or iron haematoxylin, are usually considered sufficient.

Summary

In this review I have attempted to mention and partly to explain those methods of histochemistry, in use at the present time, which can reasonably be employed by practising histologists. The accompanying photomicrographs were chosen not only to illustrate particular sections of the review but also, and this refers especially to the coloured figures, to indicate that modern histochemical techniques can produce results which bear comparison with those given by conventional staining methods (Plate I, Fig. 6). The application of the methods described to everyday problems, of functional as well as of structural pathology, can only result in increased understanding of both. It is, therefore, essential that these histochemical techniques should be applied not only by specialists, whose interests are often narrow and restricted, but by those who have access to the materials and problems of everyday pathology.

The Table on page 29 takes the place of a more conventional summary. It is a list of recommended methods for particular substances, together with the appropriate fixatives and the main references. The absence of a method from the Table does not mean that it is unworkable, and methods have been included which are useful although their specificity is open to doubt. Where many methods are available that considered best has been chosen. The references given are not necessarily those of the original author. They have been selected with a view to rapid appreciation of the technical details of the method to which they refer.

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THE NORMAL PLATELET COUNT IN MAN

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A knowledge of the concentration of blood platelets in the circulating blood is essential in the diagnosis of thrombocytopenic states and is of the greatest value in following their response to treatment.

Although the current methods employed for counting blood platelets are acknowledged to be of limited accuracy, the best of them are sufficiently accurate to be of clinical value, since only fairly gross changes in the platelet count can be related to changes in the patient's physical condition. The value of platelet counts would, however, be greatly enhanced if we had more accurate knowledge of what does in fact constitute a normal count, within what limits this is significantly determined, and what does constitute a significant change when successive counts are performed on one individual.

The normal figures for platelet counts given by various authors differ markedly according to the method used, and there is sometimes considerable variation between the results of two investigators using the same method. The methods fall into two main groups: the direct methods, in which the blood platelets are counted directly, after suitable dilution of the blood, on a haemocytometer slide; and the indirect methods, in which the proportion of platelets to erythrocytes is determined and the red cell count also, the absolute value for the platelet count being calculated from these two observations.

The mean platelet count in normal persons, according to Wintrobe (1946), is about 250,000 per c.mm. of blood. The results of some investigators are shown in Table I.

TABLE I

PLATELET COUNTS* IN HEALTHY ADULTS

Year	Author	Method	No. of Subjects	Sex of Subjects	Mean	Range†
1912	Fonio	Indirect	30	♂	234	130–350 a
1922	Kristenson	Direct	18	♂ + ♀	300	188–349 a
1926	Van Goidsenhoven	Direct	x	x	x	300–350 a
1931	MacKay	Indirect	6	x	390	250–450 a
1932	Dameshek	Indirect	52	♂	716	500–900 a
1935	Olef	Indirect	38	♂ + ♀	514	437–586 a
1935	Lempert	Direct	x	x	x	250–350 a
1936	Tocantins	Direct	40	♂	250	243–257 b
1946	Aggeler, Howard, and Lucia	Direct	64	x	409	273–545 b
1947	Masure	Direct	100	♂ + ♀	269	162–376 b

* Counts in thousands of platelets per c.mm. of blood.

† a = actual range; b = physiological range as calculated (M ± 2σ); x = not mentioned.

In this investigation platelet counts were performed on a group of healthy young adults under carefully standardized conditions in order to find the mean platelet count and the range. A second group was investigated under less standardized conditions to find whether the mean and the range of this group were significantly different from those of the control series, and to establish a normal standard for healthy young adults.

In an attempt to analyse the variability of successive counts on the same individual, two further groups were investigated in order to find the daily and day-to-day variations respectively in the platelet count.

Finally, in an attempt to assess the observer component of the experimental error, a series of diluted bloods was counted independently by three observers.

Choice of Method

For this investigation a technique was sought which would cause the minimum inconvenience to the patient, would be sufficiently rapid to permit the physician or haematologist to perform it himself, and of an order of accuracy comparable with that of other techniques.

To cause the minimum discomfort to the patient, it was decided to perform the counts on capillary blood. This has the added advantage of not requiring paraffin- or oil-coated apparatus. The capillary blood was obtained from the palmar surface of a finger-tip.

A direct method was chosen in preference to an indirect one, since the indirect methods take longer and are probably less accurate owing to the additional source of error in the red cell count itself (Biggs and Macmillan, 1948).

Three direct methods were compared in preliminary tests; the method of Rees and Ecker (1923) in which the red blood corpuscles are preserved, and the methods of Lempert (1935) and of Baar (1948) in which the red cells are haemolysed.

The method of Rees and Ecker was chosen as the most suitable for clinical use. It has the following advantages: (1) Simplicity, for only one stock solution is required, and this keeps in good condition for at least six months if stored in a refrigerator; (2) rapidity, because a platelet count by this method takes 25 minutes from start to finish (Barr's method is more rapid, but Lempert's takes longer); (3) minimum of artefacts, because there are fewer artefacts than are found with either Baar's or Lempert's methods, or any other methods which involve laking of the red cells and produce highly refractile bodies of the size of small platelets (Rees and Ecker, 1923), a source of error minimized by avoiding haemolysis; (4) other cell counts, for an additional advantage is that a red cell count may be performed on the same preparation during the time allowed for the platelets to settle (Rees and Ecker, 1923). The leucocytes also are preserved and may be counted, but the dilution is too great for this count to be of much clinical value.

Method

The composition of Rees-Ecker diluting fluid as modified by Wintrobe (1946) is shown in Table II. It is fundamentally an isotonic solution of sodium citrate, to which is added fixative and stain. The stock solution is kept in a glass-stoppered bottle in a refrigerator until required, when a small quantity is filtered into another container for current use. This

TABLE II

REES-ECKER (WINTROBE'S MODIFICATION) DILUTING FLUID

Sodium citrate	3.8 g.
Formaldehyde (neutral 40% solution)	0.2 ml.
Brilliant cresyl blue	0.05 g.
Distilled water	100.0 ml.

filtered solution is usable for the next six hours, after which the accumulation of sediment in it makes counting more difficult.

The stock solution keeps for at least six months, but samples should be filtered and examined from time to time to exclude the presence of platelet-like particles.

Pipettes and counting chambers must be scrupulously clean to avoid contact haemolysis.

The technique is as follows. Diluting fluid is drawn up to the mark 0.5 in a red cell haemocytometer pipette. The subject's finger-tip is cleaned and dried with alcohol and pricked with a Hagedorn needle, the puncture being deep enough to permit a free flow of blood. The first drop is wiped away and the second drop is drawn into the pipette until the column of diluent and blood reaches mark 1. More diluting fluid is then drawn in to the mark 101, and the pipette is shaken for three minutes.

In this investigation the further procedure was as follows. About one-third of the contents of the pipette was discarded and both sides of a double Neubauer counting chamber were filled from the remainder. To allow the platelets to settle to the same optical plane, the slide was allowed to stand from 10 to 40 minutes between two pledgets of moist cotton-wool under a petri dish to reduce evaporation. Then all the platelets in both sets of small squares were counted (a total of 800 small squares corresponding to a volume of 0.2 c.mm. of diluted blood). The reading gives the platelet count in thousands per c.mm. of undiluted blood.

The haemocytometer slides used in this investigation were to B.S.I. specification with N.P.L. certificates of accuracy to 1%. The haemocytometer pipettes were calibrated for the dilution factor and the appropriate correction made in each case.

Plan of the Investigation

The subjects for the investigation were students of physiology at Glasgow University. All were European, and their ages ranged from 18 to 29 years inclusive. No subject was included who had any history of illness or loss of blood, apart from normal menstruation, during the preceding month.

The samples were taken under standard conditions to reduce the number of physiological factors which might produce variation in the platelet count. The subject was warm and at rest in the supine position, and the sample of blood was withdrawn after a period of five minutes' recumbency.

All counts were performed by the same observer (A.W.S.), except those where two other observers performed counts on the same blood samples in order to find the difference between observers.

Results

First Series.—For the first series certain additional standard conditions were imposed in order to eliminate as many variable factors as possible. All samples were taken between 4 and 5 p.m., at least two hours after eating, smoking, or active exercise. Female subjects were not taken during menstruation or during the two days before

or after a menstrual period. A haemoglobin estimation was performed on each case, the haemoglobin being estimated as oxyhaemoglobin with a "spekker" photo-electric absorptiometer (Bell, Chambers, and Waddell, 1945), and a Leishman-stained blood film was examined for abnormal cells. Three females with haemoglobin concentrations below 12 g. per 100 ml. of blood were excluded from the series. No abnormal cells were seen in any of the remainder. This "basal" series comprises 15 men and 15 women (Table III).

TABLE III
FIRST ("BASAL") SERIES

Men*	Women	Total*
Range 140–317	Range 165–351	Range 140–351
Mean 237.9	Mean 245.3	Mean 241.7
n 14.0	n 15.0	n 29.0
σ 53.1	σ 47.1	σ 50.3
v 22.3%	v 19.2%	v 20.8%

Counts in thousands of platelets per c.mm. of capillary blood.

* = excluding 1 case with platelet countof 440,000; n = number of subjects; σ = standard deviation; v = coefficient of variation.

There is no significant difference between the mean platelet counts for men and for women in this series, and, if we exclude one quite exceptional count of 440,000 platelets per c.mm. in one of the men, we find that there is no significant difference between the range for men and for women in this series.

Second Series.—In an attempt to find the total effect of normal variations and physiological activities on the platelet count, counts were performed on a further series of 80 subjects (45 men and 35 women), the samples being withdrawn under the same conditions of warmth, rest, and posture, but in this random series the samples were taken at any time from 9 a.m. to 5 p.m., irrespective of the time after eating, smoking, or muscular exercise.

Female subjects were taken at any stage of the menstrual cycle, but careful note was taken of the exact time in the menstrual cycle when the sample was taken. In this series no further blood examination was performed, any subject being accepted who conformed to the conditions already mentioned and had no history of blood disease.

TABLE IV
SECOND ("RANDOM") SERIES

Men	Women	Total
Range 127–351	Range 165–359	Range 127–359
Mean 235.8	Mean 247.9	Mean 241.1
*n 45.0	n 35.0	n 80.0
σ 49.0	σ 51.2	σ 50.0
v 20.8%	v 20.6%	v 20.7%

Counts in thousands of platelets per c.mm. of capillary blood.

*n = number of subjects; σ = standard deviation; v = coefficient of variation.

The results of this series are shown in Table IV. No significant difference was found between the mean and range for men and for women, and there was no significant difference between the mean and range of this series and those of the basal series (excluding the one exceptional case in the basal series). The platelet counts on five of the 35 women subjects were performed during menstruation. It is of interest to note that the mean of these five readings (239,000) is not significantly different from the mean for the other 30 women (249,000).

Third Series.—In order to assess the variation in the platelet count during the day, counts were performed at standard times during the day on a series of 12 subjects (five men and seven women). The results are shown in Table V. An irregular

TABLE V

THIRD SERIES: FOUR SAMPLES FROM 12 SUBJECTS TAKEN AT STANDARD TIMES DURING THE COURSE OF ONE DAY

Subject	10 a.m.	Noon	2 p.m.	4 p.m.
1	230	235	185	239
2	213	212	165	249
3	250	308	288	259
4	154	204	202	174
5	283	214	286	236
6	319	266	248	296
7	290	288	314	271
8	131	202	158	173
9	256	237	228	286
10	255	237	259	293
11	236	217	209	192
12	244	282	256	261

Counts in thousands of platelets per c.mm. of blood.

variation was observed during the day, but this was not significant. By analysis of variance the standard error of a single observation of this series was found to be 28,000.

Fourth Series.—In order to assess the variation in the platelet count from day to day, counts were performed at the same time (11 a.m.) each day for four successive days on a series of 12 subjects (six men and six women). The women were not taken during menstruation or during the two days before or after a menstrual period (Table VI). The variation from day to day was not significant. The standard error of a single observation of this series was 15,000.

Fifth Series.—Finally, in order to assess the observer component of the experimental error, a series of 12 blood samples was examined by three observers, all experienced in haematological technique. A preparation of diluted blood was obtained in a haemocytometer pipette and was used to fill three haemocytometer slides. The counts were performed simultaneously by the three observers, each of whom retained the same slide throughout the experiment. In this experiment most variables were eliminated, leaving the distribution of platelets in the counting chambers and the difference between observers, presumably in visual acuity and in interpretation of the objects seen. The difference between observers was not significant.

TABLE VI

FOURTH SERIES: ONE SAMPLE TAKEN AT THE SAME TIME ON FOUR SUCCESSIVE DAYS FROM TWELVE SUBJECTS

Subject	1st day	2nd day	3rd day	4th day
1	306	274	312	274
2	219	199	178	221
3	270	281	282	258
4	380	322	323	334
5	310	279	283	256
6	229	219	221	218
7	270	270	264	254
8	298	311	287	298
9	204	231	202	210
10	176	184	185	163
11	325	307	326	343
12	246	244	254	252

Counts in thousands of platelets per c.mm. of blood.

TABLE VII

FIFTH SERIES: SAME BLOOD SAMPLE COUNTED SIMULTANEOUSLY BY THREE OBSERVERS

Subject	Observer 1 (A.W.S.)	Observer 2 (J.B.C.)	Observer 3 (F.O.B.)
1	181	141	164
2	154	121	155
3	210	227	217
4	217	211	230
5	223	220	183
6	299	273	278
7	293	309	346
8	227	238	238
9	183	211	197
10	268	247	242
11	142	164	166
12	232	268	192

Counts in thousands of platelets per c.mm. of blood.

Discussion

The average platelet count and the range of normal counts have been estimated by many authors, the results varying considerably with the method adopted, and in some cases there has been considerable variation between the results of two observers using the same method. Indirect methods tend to give higher counts than direct methods, but Wintrobe (1946) has pointed out that the highest counts are not necessarily the most accurate.

The finger-tip was chosen as the standard site of puncture for this investigation. Olef (1935) has pointed out that the ear is an unsatisfactory site for platelet counts, since platelets may adhere to the hairs.

A variation in platelet count with changes in posture has been claimed by von Horvath (1928) and by Steinmaurer (1932).

According to von Horvath, the platelet count falls within 15 seconds of standing up and rises on lying down. He recommended that counts should be performed from three to five minutes after the subject had assumed either the horizontal or the vertical position. Steinmaurer stated that the platelet count rose with change of posture, the greatest rise occurring when the subject stands up from lying down. For the present investigation the standard technique adopted was to take the blood sample five minutes after the subject had assumed the supine position.

Rees and Ecker (1923) recommended that the platelets should be counted 10 minutes after filling the counting chamber, since evaporation from the counting chamber alters the count after about 20 minutes. Since it is inconvenient for the clinician to be tied to a precise time, Lempert's suggestion for delaying evaporation from the counting chamber was adopted in the present investigation (Lempert, 1935). A small series of preparations, counted at 10, 30, and 50 minutes after the slide had been filled, showed at the most a quite negligible variation during this time. It was therefore assumed that, if evaporation from the slide were delayed in this fashion, the actual counting could be performed any time from 10 to 40 minutes after the slide was filled.

Rees and Ecker (1923) did not state how many squares should be counted on the haemocytometer slide. Aggeler, Howard, and Lucia (1946), using a modified Rees-Ecker technique, counted the platelets on 80 small squares of the haemocytometer chamber as for a red cell count, the figure obtained being multiplied by 10,000 to give the platelet count per c.mm. of undiluted blood. At least two chambers were counted in each case and the results averaged. A preliminary survey in the present investigation showed that, if only 80 small squares were counted in each chamber, the discrepancy between the counts on the two chambers was considerable. It was therefore considered advisable to count all the platelets in two sets of 400 small squares. The theoretical justification for counting the greater number of cells is dealt with below.

No significant difference was found between platelet counts for men and for women. This is in agreement with the conclusions of Tocantins (1938) and Wintrobe (1946) that no significant sex difference in platelet counts has ever been conclusively demonstrated.

Other physiological factors which have been claimed to influence platelet counts are race, age, exercise, alimentation, temperature, altitude, menstruation, and pregnancy. In this investigation the subjects were all European, in the same broad age group (18–29 years), and all counts were performed in the same laboratory and at a comfortable room temperature. None of the female subjects was pregnant.

For the first, or basal, series an attempt was made to eliminate the remaining known causes of variation: exercise, alimentation, time of day, and, in the female subjects, menstruation. Any immediate effect of smoking was also eliminated.

For the second, or random, series these additional criteria were not applied, it being assumed that any significant changes in the platelet counts of the group as a result of additional activities or normal daily variations would result in a difference between the mean or the scatter of this series and those of the basal series. No significant difference was found, which suggests that the platelet counts performed at

any time of the normal working day (9 a.m. to 5 p.m.) are equally valid for comparative purposes.

Taking the largest series of observations (Series 2) as the standard, the average platelet count in the 80 young adults tested by this method was found to be 241,000, and the standard deviation 50,000. This gives us a " normal range," calculated according to the usual convention as the mean $\pm$ twice the standard deviation, of approximately 140,000–340,000, and 95% of the readings did in fact fall within these limits.

Changes in the platelet count have been related to the time of day (Degkwitz) 1920; Kristenson, 1924; Kranzfeld, 1925), but Zeller (1921) and Otsuka (1933, found little difference between counts taken at different times during the day. In this investigation no significant variation was noted during the day (Table V), and supports the impression gained from comparison of the first and second series that counts performed at any time during the normal working day are equally valid for comparative purposes. The standard error of a single observation of this series (28,000) is the standard error of a single platelet count taken at any time between 9 a.m. and 5 p.m. A single platelet count performed during this period may therefore be regarded as significantly determined to $\pm$56,000 ($\pm$23%), and a difference of 80,000 (30%) between two successive counts on the same subject would be significant.

A further series (Table VI) showed no significant difference between counts performed on successive days on each subject. The day-by-day variation was even less than the daily variation, presumably because variations caused by physiological activities such as alimentation and muscular exercise were minimized. In this series (Table VI) the blood samples were all taken at 11 a.m., after the student had been sitting for one hour at a lecture. The standard error of a single observation of this series is 15,000, which, as is noted below, is approximately the theoretical minimum error for the method employed.

" Student " (1907) showed mathematically the minimum error which must be present in all counts performed with a haemocytometer, owing to the random distribution of the particles over the field. Later he indicated (" Student," 1919) that the distribution follows Poisson's law and that the probable error of a haemocytometer count is $0.6745 \sqrt{N}$, where N is the total number of cells counted. This will only be true if the presence of particles in a certain division of the field does not appreciably affect the probability of other particles entering that division. When the particles are blood cells and the suspending medium blood, diluted 1 in 200, this is believed to be the case.

Berkson, Magath, and Hurn (1939) divided the minimum error of the haemocytometer count into three parts: " field error," which depends on the Poisson distribution; " chamber error," which depends on inaccuracy of calibration of the chamber and differences in the procedure of filling the chamber; and " pipette error," which depends on inaccurate calibration of the pipette and on differences in the manipulations of filling, diluting, and mixing. They found that under the most favourable conditions, with careful technique and standardized apparatus, the field error might be practically the only effective error. Biggs and Macmillan (1948), from a series of platelet counts on one subject by five observers using five pipette and counting

chamber combinations, calculated the error due to observers, calibration of pipettes and counting chambers, and the time of taking the sample. The main sources of error were found to be the observer component and the random variation, and, with a direct method (Lempert, 1935), the random variation approached the Poisson distribution.

It is obviously important to know the field error of the haemocytometer technique in use, since this is the minimum error which would remain under ideal conditions, and the error of any method involving the use of a haemocytometer cannot be less than this. The field error depends on the Poisson distribution and is proportional to the square root of the number of cells counted. The percentage field error therefore varies inversely as the square root of the number of cells counted. The percentage field error of a count can therefore only be reduced by counting more cells. In this investigation (Series 2) an average number of 241 cells was counted (on 2 sq. mm. of haemocytometer field). For this number, the field error of the platelet count from the Poisson distribution would have a standard deviation of approximately 15,000, and this is the minimum which can be hoped for by this method. The error could, however, be reduced by performing more than one count on each occasion.

By calibration of pipettes and counting chambers and standardization of technique an attempt was made to reduce pipette and chamber error to the minimum. The observed variations from day to day (Series 4) approximate to the inevitable variation dependent on the Poisson distribution, and support the view that this is a good method.

Finally the difference between observers was investigated. Some authors (Ivanitzky-Vasilenko and Klimova, 1937) find no significant difference between counts performed on the same subject by two observers, whereas others (Aggeler *et al.*, 1946) maintain that the difference is so great that it is impossible to obtain similar results from different technicians and that consequently each must establish his own " normal range." As observers can be taught to withdraw blood, fill the pipette and mix the contents, and fill the counting chamber according to a standardized technique, it was felt that the most important factors in determining the observer component of the error must be the visual acuity of the observers and their interpretation of the objects seen. This was accordingly investigated, and no significant difference was found between counts made by different trained observers on different portions of the same sample of diluted blood. From this it is assumed that reasonable agreement can be found between platelet counts performed by different trained observers, using the same technique and working under similar conditions.

Summary

By a modification of the method of Rees and Ecker (1923) the mean platelet count on 80 healthy young adults was found to be 241,000 and the " normal range " ($M \pm 2\sigma$), approximately 140,000–340,000.

Between these limits a count is significantly determined to $\pm$56,000 ($\pm$23%).

No significant difference was found between the platelet counts on men and those on women.

The variation in the platelet count of individuals during the day and from day to day was not significant.

In successive samples from one subject in this range, taken at any time between 9 a.m. and 5 p.m., a change of not less than 80,000 (30%) in the platelet count is significant, whether the counts have been performed by the same observer or by different trained observers.

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PLASMACYTOSIS IN THE BONE MARROW IN RHEUMATOID ARTHRITIS

BY

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A woman suffering from severe rheumatoid arthritis was found to have a hypochromic microcytic anaemia. The anaemia did not respond to iron by mouth but was treated satisfactorily by intravenous iron. A sternal marrow examination before beginning intravenous iron therapy showed several clumps of up to seven or eight plasma cells, and a percentage of plasma cells amounting to 4.8 in 1,000 marrow cells counted. Many of these plasma cells were vacuolated, with irregularity of the cytoplasmic basophilia, and an occasional plasmablast was seen. Multiple myelomatosis was considered as a possible diagnosis, but further clinical examination and investigations excluded this condition. It was concluded that the finding was incidental, though perhaps related to rheumatoid arthritis.

Numerous studies of the sedimentation rate, colloidal gold tests, and plasma viscosity have shown a disturbance in the serum protein fractions in rheumatoid arthritis, and Perlmann and Kaufman (1946) by electrophoretic analysis of the sera from 23 cases showed that the alpha globulins were raised early in the disease and the gamma globulins later. They suggested that articular and synovial damage may liberate a pathological protein similar in molecular weight and electrophoretic mobility to the gamma globulins.

A more likely theory which has been put forward to explain the gamma globulin increase is that an unknown antigen may act as a stimulus for antibody formation. This is based on the known association between some antibodies and the gamma globulin fraction (Enders, 1944).

Whatever the mechanism of stimulation, the possibility that a marrow plasmacytosis might be found in association with the gamma globulin increase was suggested by the considerable volume of work (Bing and Plum, 1937; Kagan, 1943; and many others) which has established a connexion between plasma cells, antibody formation, and hyperglobulinaemia. Much of this work is reviewed by Fagraeus (1948).

It therefore seemed worth finding out whether the plasmacytosis observed in the marrow of our original case was common in rheumatoid arthritis. A series of ten cases of rheumatoid arthritis encountered consecutively and unselected was therefore investigated, and sternal marrow examinations showed that in every case the plasma cell content was increased beyond the usually accepted normal figures.

Methods

Preparation of Bone Marrow Smear.—Not more than 0.2 ml. of marrow was aspirated from the manubrium or body of the sternum through a Salah needle. Smears were made direct on to clean glass slides and stained by Leishman's method. The error of total cell counts on bone marrow aspirates is so great that they were not performed, but all specimens showed normal cellularity as judged by the cell density in stained films.

Plasma Proteins.—Plasma proteins were estimated by the micro-Kjeldahl technique. The total protein, albumin, globulin, and fibrinogen fractions were determined.

Sedimentation Rate.—The red cell sedimentation rate was measured on oxalated venous blood by Wintrobe's method. The rate was corrected for anaemia using the correction chart of Hynes and Whitby (1938).

Classification of Plasmacytic Elements

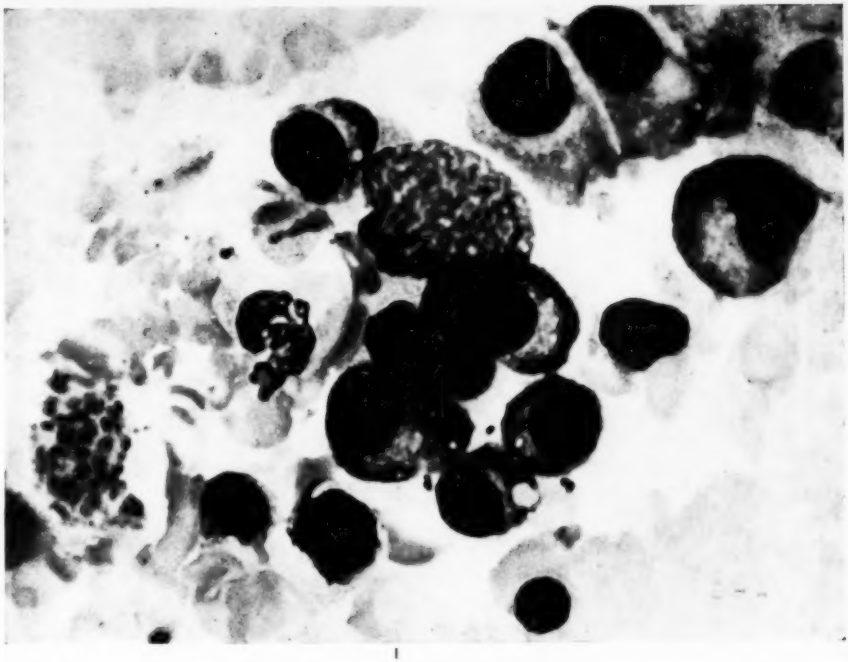
The classification of the plasmacytic elements closely follows that used by Fadem and McBirnie (1950), based on the recommendations of " The Committee for the Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood-forming Organs," and the descriptions below are taken, with minor modifications, from their paper. The degenerating plasmacytes have, however, been further subdivided into cells showing " early homogenous degeneration " and " degenerative plasmacytes " (Fig. 1). Michels (1931) recorded five types of degenerative changes occurring in plasma cells, but this detailed classification was considered unnecessary in the present investigation.

Plasmablast.—The plasmablast varies from 20 to 40 μ in diameter. The nucleus is large, 15–30 μ in diameter, and is surrounded by a thin rim of cytoplasm. Its nuclear chromatin is fine and reticular, and there may be one to three pale, blue-staining nucleoli distributed irregularly in the nucleus. There is no nuclear membrane, but the chromatin fibrillae are distributed radially at the periphery of the nucleus. The cytoplasm occasionally contains irregularly sized, round, non-staining vacuoles (Fig. 3).

Proplasmacytes.—The proplasmacyte varies from 15 to 30 μ in diameter. Its nucleus may be centrally or eccentrically placed within the cell. The nuclear chromatin is coarsely reticular and irregularly clumped. A nuclear membrane is suggested at this stage by clumping of the chromatin at the periphery of the nucleus. Nucleoli are absent. The cytoplasm is more abundant in relation to the size of the nucleus than that of the plasmablast. The cytoplasm is basophilic and stains with different intensities of blue from light to dark. Non-staining, irregularly sized round vacuoles are frequently found in the cytoplasm.

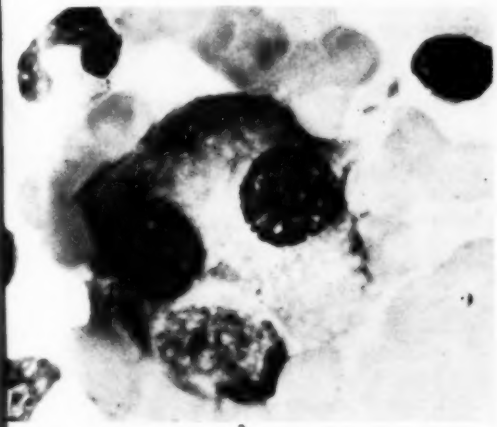
Plasmacyte.—The plasmacyte is a cell 8 to 25 μ in diameter that has a relatively large amount of cytoplasm in relation to the size of the nucleus. The nucleus is in rather an eccentric position within the cell, and nuclear chromatin is clumped in an irregular pattern that often resembles a " spoke-wheel."

The definite nuclear membrane appears to be the result of heavy clumping of the chromatin at the periphery of the nucleus. Binucleated and trinucleated plasmacytes are observed. The cytoplasm is diffusely basophilic and stains a light to medium blue. Frequently a perinuclear, clear, non-staining zone is present in the cytoplasm.



1. Group of seven plasmacytes, two "early homogenous degeneration" cells, and a reticulum cell from Case VII. × 1500.

2. Binucleated plasmacyte from Case VI. × 1500.



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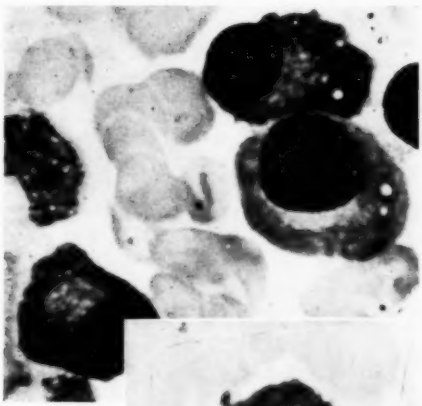

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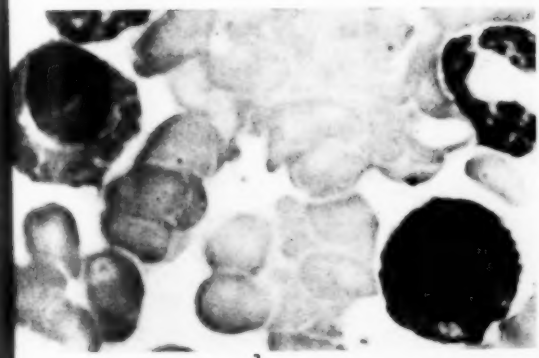

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3. Plasmablast (right) and mature plasmacyte (left) from Case VIII. × 1500.

4. Group of three plasmacytes, one showing early homogenous degeneration from Case VI. × 1500.

5. Plasmacyte showing distortion of cytoplasmic outline from Case VII. × 1500.

Irregularly sized, round, non-staining vacuoles are frequently found in the cytoplasm and are most abundant at the periphery of the cell.

Plasmacyte Showing Early Homogenous Degeneration.—The nucleus remains intact as in the plasmacyte, but the cytoplasm is characterized by uneven light staining. The cell outline remains well defined (Fig. 4).

Degenerative Plasmacytes.—These represent a more advanced state of degeneration than the previous type. The nucleus is pyknotic with early fragmentation. The cytoplasm is less well stained and frequently has a foamy appearance. The cell outline is often indistinct.

Total and Differential Count of the Plasmacytic Elements.—The total percentage of cells of the plasmacytic series was obtained from a count of 1,000 bone marrow cells from each case. The differential count of the plasmacytic elements was obtained by counting 100 cells of the plasmacytic series from each case, and in addition the number of binucleated and trinucleated cells and cells showing mitotic figures was noted.

Case Material and Results

The ten patients in this series were all suffering from rheumatoid arthritis as judged by the usual clinical criteria, and covered a wide range in age, duration of disease, and severity of present symptoms. These details are included in Table I, together with total and differential plasma cell percentages in the marrows, figures for plasma protein estimations, and sedimentation rates. Table II provides the complete differential counts of 1,000 bone marrow cells from each case, showing normal cellular distributions with the exception of the plasmacytosis.

Discussion

The Normal Level of Plasma Cells in Sternal Marrow.—Normal differential counts on human sternal marrow published from 13 authoritative sources are summarized by Bodley Scott (1939), and the plasma cell counts in these series give an overall range from 0 to 1.1% ; Whitby and Britton (1950) give a range from 0 to 1% and Custer (1949) from 0 to 1.2%. A level of 2% or over may certainly be taken as outside the normal maximum.

Bone Marrow Plasmacytosis in Disease.—An increase in plasma cells in the marrow in conditions other than multiple myeloma, plasma cell leukaemia, and diffuse plasma cell myelosis has been noted by a number of authors (Bayrd, 1948 ; Bing, 1940 ; Falconer and Leonard, 1948 ; Kolff and Dhont, 1948) in a wide variety of conditions. These include granulomas and chronic infections, measles, roseola infantum, carcinoma, aplastic anaemia, infectious mononucleosis, monocytic leukaemia, Boeck's sarcoid, cirrhosis, lymphogranuloma inguinale, kala-azar, polyarteritis nodosa, Hodgkin's disease, and some acute infections. The reports are summarized by Fadem and McBirnie (1950), who describe a further six cases with from 5.4 to 23.6% of plasma cells in the marrow. In their cases, Hodgkin's sarcoma, lymphosarcoma, acute monocytic leukaemia, primary " refractory " anaemia, hiatal hernia, and papilloma of the bladder, the plasmacytosis was encountered during the examination of 100 routine bone marrow preparations. The increased percentage of plasmacytic elements in these conditions is not, however, a constant or even a frequent

TABLE I

CLINICAL AND LABORATORY DATA ON TEN CASES OF RHEUMATOID ARTHRITIS IN THIS SERIES

Case No.	I	II	III	IV	V	VI	VII	VIII	IX	X
Sex	F	F	F	F	F	M	M	F	M	F
Age (years)	30	81	73	68	47	27	56	24	53	42
Duration of disease	1 yr.	11 yrs.	28 yrs.	10 yrs.	8 yrs.	4 mths.	1 yr.	1½ yrs.	1½ yrs.	4 yrs.
Severity	severe	moderate	moderate	severe	moderate	severe	moderate	moderate	severe	mild
Total plasma cell %	4.6	6.3	4.0	3.4	2.2	3.6	5.1	5.5	3.9	1.8
Differential count of plasmacytic element*										
Plasmablast	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	1.0
Proplasmacyte	1.0	0.0	0.0	0.0	4.0	1.0	4.0	0.0	1.0	3.0
Plasmacyte	77.0	74.0	84.0	71.0	71.0	67.0	77.0	74.0	85.0	73.0
Early homogenous degeneration	16.0	20.0	10.0	24.0	16.0	25.0	16.0	22.0	12.0	19.0
Degenerative plasmacyte	6.0	6.0	6.0	5.0	9.0	7.0	3.0	3.0	2.0	5.0
Binucleated plasmacyte	8.0	1.0	0.0	6.0	1.0	3.0	0.0	1.0	1.0	0.0
Trinucleated plasmacyte	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Plasmacyte in mitosis	1.0	1.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	1.0
Plasma proteins										
Total (g./100 ml.)	—	7.2	7.0	8.2	9.75	7.7	—	8.0	—	—
Albumin (g./100 ml.)	—	3.65	3.3	2.9	4.3	3.2	—	3.25	—	—
Globulin ,, ,,	—	3.35	3.4	4.8	5.1	4.15	—	4.35	—	—
Fibrinogen ,, ,,	—	0.4	0.26	0.42	0.35	0.35	—	0.4	—	—
E.S.R. (mm. in first hour)	31	28	2	2	14	28	31	37	16	40

* This differential was obtained by counting 100 consecutive plasmacytic elements in each marrow smear.

TABLE II

DIFFERENTIAL COUNTS OF 1,000 BONE MARROW CELLS FOR EACH CASE IN THIS SERIES

Case No.		I	II	III	IV	V	VI	VII	VIII	IX	X
Polymorphs	Neutrophils	22.7	25.5	37.4	10.5	24.1	21.0	18.4	12.5	32.8	17.2
	Eosinophils	1.5	1.0	0.9	1.2	0.2	1.6	1.4	1.0	1.4	1.7
	Basophils	0.1	0.1	0.3	0.2	0.2	0.3	0.3	0.0	0.2	0.3
Metamyelocytes	Neutrophils	17.5	18.9	12.6	16.4	15.6	16.2	17.5	16.5	18.8	25.0
	Eosinophils	1.2	1.0	0.5	1.6	0.7	2.0	1.3	1.0	1.7	2.5
	Basophils	0.0	0.1	0.1	0.0	0.1	0.0	0.0	0.2	0.0	0.0
Myelocytes	Neutrophils	14.3	17.0	11.2	19.1	17.2	12.2	19.3	13.0	12.8	16.1
	Eosinophils	1.9	1.7	0.5	2.4	1.0	2.2	3.2	1.0	1.3	3.0
	Basophils	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Promyelocytes		1.2	1.0	0.5	1.1	1.2	1.7	1.0	2.0	0.5	1.8
Myeloblasts		0.7	0.9	0.2	0.4	0.3	1.2	0.7	1.5	0.3	0.5
Lymphocytes		5.9	6.7	11.8	14.7	14.9	5.8	5.6	26.0	6.4	12.7
Monocytes		0.4	0.6	1.9	1.0	0.0	0.3	0.4	0.5	0.5	0.5
Plasma cells		4.6	6.3	4.0	3.4	2.2	3.6	5.1	5.5	3.9	1.8
Normoblasts	Late	15.8	14.9	11.9	15.1	13.2	18.5	17.4	13.0	15.1	8.7
	Intermediate	5.5	2.8	4.7	9.1	7.2	8.1	4.7	5.5	2.6	2.7
	Early	5.7	0.9	1.5	2.6	1.5	3.3	1.5	1.0	0.8	1.7
Proerythroblasts		1.0	0.2	0.0	0.7	0.4	1.1	1.5	0.0	0.7	1.5
Haemocytoblasts		0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.3
Reticulum cells		0.0	0.5	0.0	0.4	0.0	0.0	0.7	0.0	0.2	0.0

finding, and with the exception of Fadem and McBirnie's report no figures are given of the level of plasmacytosis. No specific reference is made in any of these papers to rheumatoid arthritis, although "chronic infections" are mentioned as associated sometimes with an increase in plasma cells.

The present investigation has shown a remarkably constant increase in the plasma cell content of the marrow in rheumatoid arthritis, figures ranging from 1.8 to 6.3% being encountered. This finding serves to emphasize that a diagnosis of multiple myelomatosis cannot be based solely on an increase of bone marrow plasma cells over 3%, but must be supported by other evidence. Clumps of plasma cells, up to ten in number, vacuolation, cytoplasmic irregularities of shape and staining, multinucleated cells, and various stages of degeneration, all features which are sometimes regarded as characteristic of myeloma cells, were observed in our material (Figs. 2 and 5).

The plasma samples upon which protein estimations were carried out showed a definite increase in globulin. Although the inaccuracies of the micro-Kjeldahl method render the assessment of individual figures difficult, the constancy of this globulin elevation appears to be significant. More accurate methods in the hands of other investigators have shown that the gamma globulins in rheumatoid arthritis are usually increased (Perlmann and Kaufman, 1946 ; Lövgren, 1945 ; Malmros and Blix, 1946), and the marrow plasmacytosis which we have observed may throw some light on the source of this increase. The hypothesis that foreign proteins are produced at the sites of joint damage becomes less likely if the plasma cells in the marrow are responsible for globulin formation. The results in Table I show that the plasmacytosis and hyperglobulinaemia are not dependent upon the presence of active lesions, since they occur whatever the degree of activity judged by the sedimentation rate. It seems more probable that the increased protein production is a generalized, systemic response, possibly to a circulating antigen, rather than a localized reaction. Support for this conception is lent by the observations of Kolouch (1938), who demonstrated a marked increase in the number of plasma cells in the bone marrow of rabbits, sensitized against *Streptococcus viridans* during a period when the antibody titre was rising.

Our observations, therefore, favour the view that the hyperglobulinaemia of rheumatoid arthritis is an antibody response, with accompanying plasmacytosis, to an unknown antigen.

Summary

Ten cases of rheumatoid arthritis showed an increase in bone marrow plasma cells, ranging from 1.8 to 6.3%.

Attention is drawn to the possibility of confusion between the marrow pictures in multiple myeloma and rheumatoid arthritis.

The relationship between hyperglobulinaemia and plasmacytosis is briefly discussed.

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EMERGENCY METHODS FOR RHESUS GROUP DETERMINATION

BY

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The most reliable method for Rhesus group determination is probably the standard tube method (Landsteiner and Wiener, 1941; Boorman, Dodd, and Mollison, 1942; Taylor, 1943). This technique has only one serious disadvantage: it may take as long as two hours to perform, although occasional high-avidity anti-D sera will give clear-cut results in 30 minutes or even less. For emergency Rhesus typing a considerably quicker procedure is desirable. Rapid methods which have been described include the Chown capillary tube test (Chown, 1944 ; Chown and Lewis, 1946) and the Diamond slide test (Diamond and Abelson, 1945). Mollison, Mourant, and Race have recommended centrifuging after ten minutes' incubation as a means of accelerating agglutination in the tube method.

In pre-transfusion Rhesus grouping in this laboratory, it has been the practice for some time to use the Chown method. The results were checked by the tube method carried out by a different observer working in a separate room. Where both tests were in agreement, it was assumed that the correct result had been found: whenever discrepancies occurred, investigations were repeated until no reasonable doubt remained. After several hundred parallel observations had been made in this way, it became clear that the Chown test, at any rate with the technique in use, was not altogether satisfactory: both false positive and false negative results were occasionally encountered. We therefore decided to carry out a number of experiments to try to discover the reasons for incorrect results in the Chown test, and to determine as far as possible the optimum conditions for the test. At the same time a trial of the tube centrifuging method was made in parallel with the other two techniques.

We aim in this paper to compare the reliability of three methods for Rhesus group determination as used in a routine laboratory ; to draw attention to some sources of error in the Chown test ; and to describe the conditions in which satisfactory results can be obtained with the tube centrifuging method.

Methods

The Chown Test.—A thick suspension of cells in their own serum is obtained by shaking up the remains of a clotted specimen of blood after most of the serum has been removed. This is allowed to fall through a column of anti-D serum in a capillary tube set up in a small lump of plasticine and inclined at an angle of 45°. Approximately equal volumes of cell suspension and anti-D serum are used. The test is carried out at room temperature and read after 15 minutes. This technique is substantially the same as that advocated by Mollison *et al.* (1948). Standard vaccine capillary tubes have been used in most of the experiments, as

these are of convenient size and approximately uniform bore (0.7 to 0.9 mm.). In some of the experiments these conditions have been varied.

The Tube Method.—One drop of a 2% cell suspension in citrate saline (0.5% sodium citrate, 0.85% sodium chloride) is mixed with one drop of anti-D serum in a $2\frac{1}{2} \times \frac{3}{8}$-in. test-tube and placed in an incubator at 37° C. The results are read after two hours.

The Tube Centrifuging Method.—The cell suspension and anti-D serum are mixed in a small test-tube as before. The tube is placed in a 37° C. water bath for 10 minutes and is then centrifuged for five minutes at 2,000 to 2,500 r.p.m. The test is read by rotating the tube gently between the thumb and forefinger : a positive result is indicated by a mass of agglutinated cells which becomes detached from the base of the tube and is surrounded by clear fluid ; if the result is negative, the cells, when rotated, will spiral upwards in supernatant fluid. It is sometimes necessary to shake the tube gently after rotation in order to see the result clearly, but vigorous shaking should be avoided, as it tends to break up the agglutination.

Results

In 917 consecutive routine determinations of the Rhesus group by the Chown method in parallel with the standard tube method, 20 discrepancies occurred. In each case the Chown test was found to be at fault : there were nine false positive and 11 false negative results. In the last 360 of these observations, the tube centrifuging method was used in addition to the other two tests. No discrepancies between the standard tube method and the tube centrifuging method arose, but the Chown test gave three false positive and four false negative results. In a further 180 observations the standard tube method and the tube centrifuging method were in complete agreement. Thus 540 consecutive observations were performed by the tube centrifuging method without error.

Factors Affecting the Reliability of the Chown Test

Experiments designed to investigate the various factors influencing the success or failure of the Chown test were carried out as described below. Several different anti-D sera were used in the different experiments, but in any one experiment the same serum was always used.

Temperature.—Previous workers have recommended incubation of the capillary tubes at 37° C., and this seems logical since agglutination with Rhesus antibodies is known to take place most rapidly at this temperature. However, it is more convenient to perform the test at room temperature, and we find that agglutination at room temperature is not in fact much slower. Table I shows the time taken for the test to become positive at 19° C. and at 37° C. for ten blood samples.

A disadvantage of incubation lies in the fact that at 37° C. the agglutinated clumps of cells in a positive reaction fall more rapidly to the bottom of the capillary tube and become packed together. Finally, the appearance differs very little from that of a

TABLE I

TIME REQUIRED FOR POSITIVE RESULTS IN TEN SAMPLES AT TWO TEMPERATURES

Temperature	Average Time to Reach Positive Result	Shortest and Longest Times	
19° C.	9′ 0″	7′ 20″	13′ 30″
37° C.	6′ 50″	4′ 40″	8′ 50″

negative test left for a similar period. At room temperature there is a longer period during which positive and negative results are easily distinguishable.

Diameter of Capillary Tubes.—Fifty observations were made using capillary tubes of internal diameter varying from 0.3 to 1.4 mm. The times taken for a positive result to appear are shown in Fig. 1. There is evidently no correlation between the two factors over the range of sizes used in this experiment. There is, however, a considerable difference in the clarity of the results, those in the largest and smallest sizes in this range being much less distinct than those in the intermediate values. An internal diameter of 0.6 to 0.9 mm. seems to give the best results.

Effect of Rouleau Formation in Producing False Positives.—In a number of instances in which false positive results were obtained, it was noticed that the patients were suffering from conditions likely to give rise to a raised erythrocyte sedimentation rate (E.S.R.), and it therefore seemed probable that the apparent agglutination obtained in these cases might in fact be due to rouleau formation. It was soon found that a cell suspension made in the way described and drawn into a capillary tube supported at an angle of 45° would rapidly give the appearance of a positive reaction when no anti-D serum had in fact been added. The macroscopic appearance was indistinguishable from that of a true positive result, and a check by microscopic examination was not possible as the red cell clumps dispersed in either case on transferring them to a slide.

In order to discover the conditions under which false positive reactions arose, we selected a number of blood specimens from patients known to have a high E.S.R. Table II shows the reactions obtained with and without anti-D serum in seven

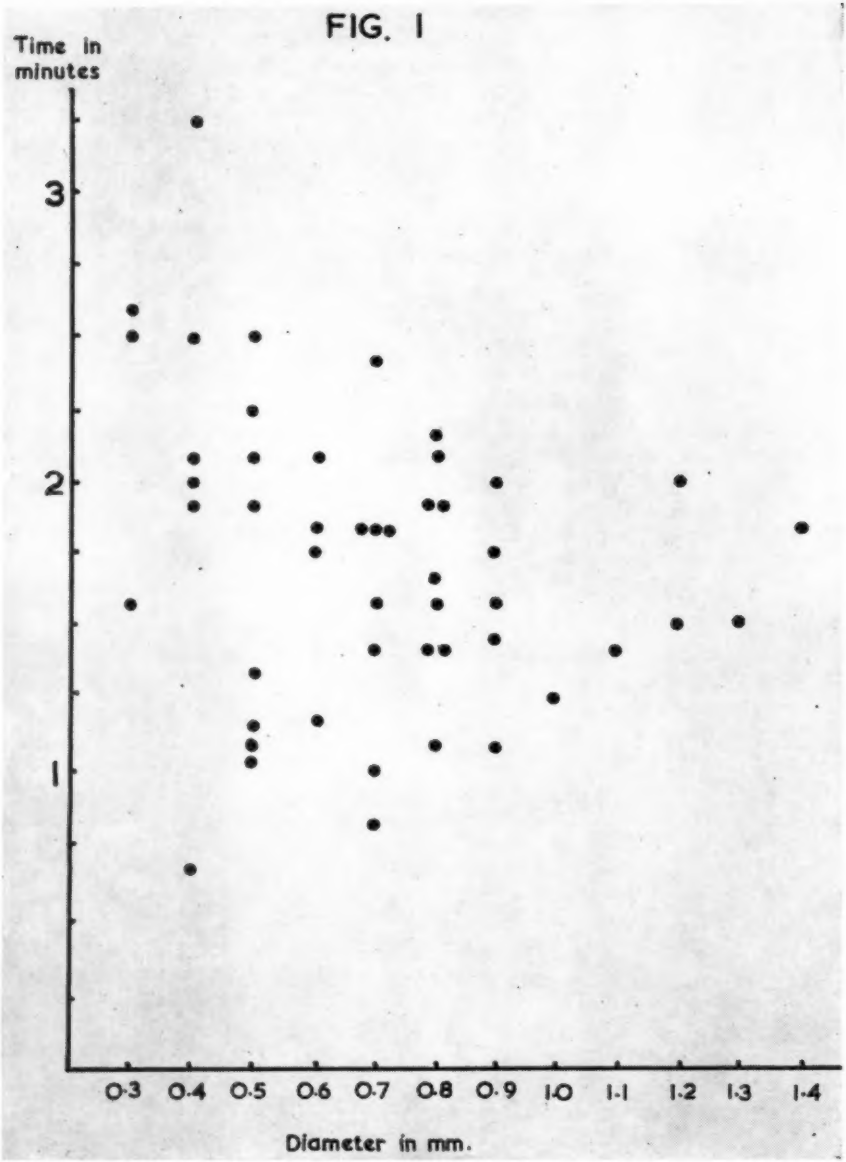
TABLE II

ANALYSIS OF REACTIONS WITH AND WITHOUT ANTI-D SERUM IN SEVEN RH-POSITIVE PATIENTS

E.S.R. (mm. in 1 hour Westergren)		Dilution of Packed Cells in their Own Serum					
		40%	30%	20%	10%	5%	2.5%
89	No anti-D serum	++	++	+++	+++	++	−
	Anti-D serum added	++	++	+++	+++	+++	±
59	No anti-D serum	+	++	++	++	+	−
	Anti-D serum added	++	+	+++	+++	+++	+
45	No anti-D serum	++++	++++	++++	++++	++	+
	Anti-D serum added	+++	++	++	+++	+++	++
105	No anti-D serum	++	++	++	+++	++	++
	Anti-D serum added	++	++++	++++	+++	+++	+++
45	No anti-D serum	−	+	+	++	++	+++
	Anti-D serum added	++	++	+++	+++	++	++
65	No anti-D serum	+	+	+	++	+	+
	Anti-D serum added	+++	++++	++++	+++	+++	+++
67	No anti-D serum	+	+	++	++	+	+
	Anti-D serum added	++++	++++	++++	++++	+++	+++

++++ = clear spaces more than 1 mm. wide between the red cell clumps ; +++ = clear spaces less than 1 mm. wide between the red cell clumps ; ++ = the red cells form a continuous column, but clumping evident ; + = a definite irregularity of the red cell column ; ± = a barely perceptible irregularity of the red cell column ; − = the red cell column shows no irregularity.

FIG. 1


Time in minutes
3
2
1
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
1·1
1·2
1·3
1·4
Diameter in mm.

Rhesus-positive patients at varying dilutions of cells in their own sera. The blood specimens were first centrifuged and appropriate dilutions of packed cells in serum were prepared.

These results make it clear that the Chown test, using a suspension of red cells in their own serum, is not trustworthy in patients with a high E.S.R. In order to test the effect of different diluting fluids, we made 192 observations with cell suspensions from three of these blood samples. Eight different concentrations of cells were used, ranging from 40% to 0.5%. They were diluted with (1) their own serum ; (2) serum from a healthy subject of group AB ; (3) citrate saline ; and (4) citrate saline using cells which had been washed three times. It was found that by using citrate saline suspensions false positive results due to rouleau formation could be avoided. There seems to be no advantage in washing the cells previously. With AB serum, however, false positive results can still be obtained, though they are generally weaker than those which appear when suspensions of cells in their own serum are employed. Citrate saline suspensions give much less clearly marked positive results than those obtained with serum suspensions, and in high dilution false negative results may be obtained.

Effect of Dilution in Producing False Negatives.—The speed at which a positive result is obtained depends to a varying extent on the concentration of the cell suspension used. Table III shows the times taken in minutes for a positive result to appear when ten blood samples were diluted to a varying degree with their own serum.

TABLE III

TIMING (MINUTES) OF POSITIVE RESULT IN VARYING DILUTIONS OF TEN BLOOD SAMPLES

Blood Sample	Dilution							
	1 : 1	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128
1	3	5	5	7	7	10	9	6
2	4	3	4	5	7	6	7	7
3	3	3	4	4	4	5	4	5
4	3	3	4	4	6	6	6	6
5	2	2	4	3	3	2	2	2
6	4	3	3	2	3	4	4	4
7	5	7	10	7	10	9	8	9
8	4	3	3	6	7	7	7	10
9	2	10	11	12	13	11	10	9
10	7	14	14	14	14	14	14	13

The delaying effect of dilution is much more marked with citrate saline suspensions. Table IV shows the time in minutes taken for the test to become positive when varying dilutions in citrate saline are employed.

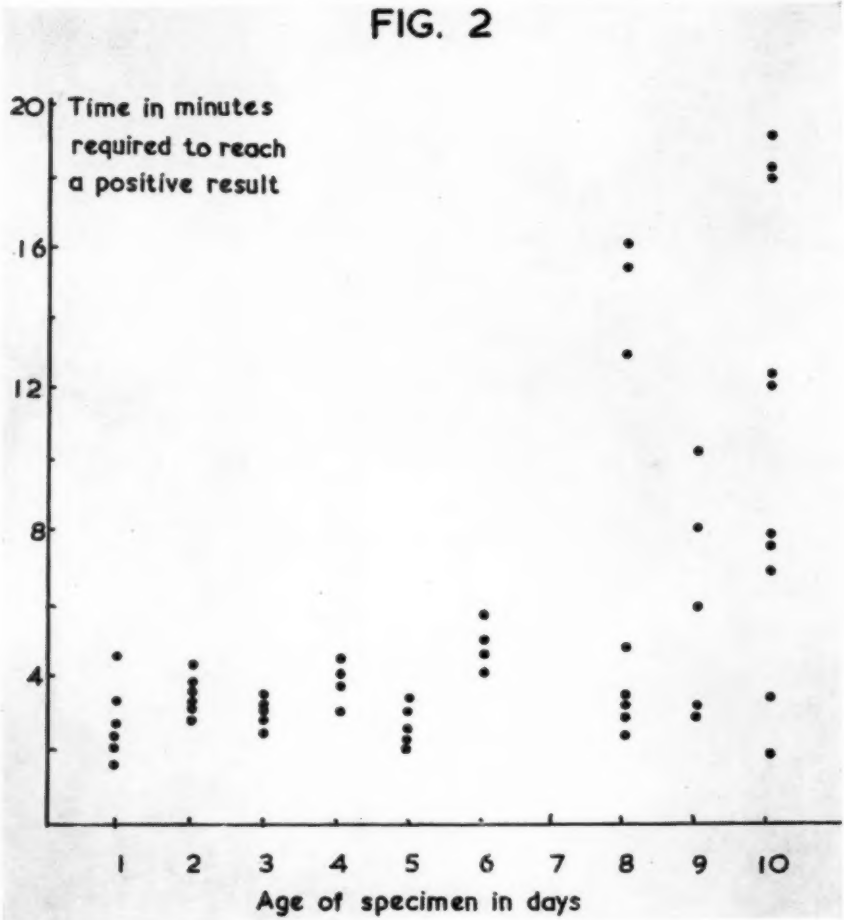
Effect of Age of the Blood Sample Tested.—Discombe and Meyer (1948) drew attention to the fact that blood samples which are not fresh may give false negative results. In Fig. 2 the times taken to reach a positive result are shown for 55 blood samples, known to be Rhesus-positive, which had been collected up to 10 days previously. A positive result was eventually obtained in every case. It will be seen that for blood samples up to five days old no great difference is apparent. After that,

TABLE IV

TIMING (MINUTES) OF POSITIVE RESULT IN DILUTIONS WITH CITRATE SALINE IN SEVEN BLOOD SAMPLES

Blood Sample	Dilution								
	1 : 1	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256
1	2	7	8	15	27	24	35	38	61
2	7	9	10	11	18	18	23	25	27
3	5	10	11	14	17	29	29	35	38
4	4	9	11	15	15	17	17	29	31
5	3	6	10	12	17	26	26	34	81
6	3	7	11	13	14	14	21	22	28
7	3	7	8	13	17	31	40	43	43

FIG. 2



however, some show considerable delay in the development of a positive reaction, although others give results after times comparable with those of the fresher specimens.

Conditions for Satisfactory Results by the Tube Centrifuging Method

As has been shown, reliable results can be achieved by this method if the tubes are incubated for 10 minutes and then centrifuged for five minutes. These times cannot be shortened to any great extent without sacrificing the reliability of the test. There is, of course, no objection to prolonging either time.

It was feared at first that prolonged centrifuging might, in a negative reaction, pack the cells so tightly together that false positive results could occur. Accordingly five tests were set up with known Rhesus-negative blood samples and centrifuged for 30 minutes. All were clearly negative at the end of this time. In five more tests, with 60 minutes' centrifuging, all results were again obviously negative.

In order to test the validity of the tube centrifuging method with anti-D sera of varying titre, ten blood samples, seven Rhesus positive and three Rhesus negative, were tested with each of five different antisera of titre varying from 1 : 2 to 1 : 32. Five different observers, who had no previous experience of the test, were asked to read the results. A sixth observer determined the Rhesus groups by the standard tube method. No discrepancies were found.

Discussion

The extent of error which we have found in the routine use of the Chown test (20 discrepancies in 917 consecutive observations) is approximately the same as that encountered by Discombe and Meyer (1948). These authors examined 403 blood specimens ; three were rejected as unsatisfactory specimens, although two of the three gave adequate results with the tube method. A further eight results are described as doubtful. Thus, in 10 out of 402 cases which gave satisfactory results by the tube method, they found the Chown test inadequate. Discombe and Meyer's technique differs from ours in that they used saline suspensions of cells and incubated their capillary tubes at 37° C.

The most serious source of error in the Chown test seems to be the occurrence of false positive results due to rouleau formation when serum suspensions of cells are used. False negative results are, on the other hand, apparently caused by a variety of factors which delay the appearance of a positive result beyond the time selected for reading the test. One of these factors is dilution, which delays the appearance of a positive reaction both with serum and with saline suspensions, but to a much greater extent with saline suspensions. Another delaying factor is the age of the specimen of blood : with specimens over five days old considerable delay may occur, but up to this time little difference can be detected.

False positive results due to rouleau formation can be avoided by the use of saline suspensions, but if this modification is adopted there will be an increased risk of false negative results if some of the cell suspensions are unduly weak. To centrifuge the cells and then dilute them accurately is undesirably tedious in a routine laboratory procedure. Great prolongation of the time of the test is dangerous, as in rapidly reacting specimens the cells quickly fall to the bottom of the tube and the test becomes difficult to read. All sources of error could probably be avoided by using a 20%

suspension of cells in citrate saline and observing the test every few minutes for half an hour. If this is to be done, the Chown test is by no means a simple and rapid technique, and its principal advantages disappear.

The Diamond slide test is a rapid method of which we have no personal experience. Its authors state that rouleau formation is a frequent complication. We believe that any test which depends for its success on the certain differentiation of agglutination from rouleau formation is liable to give rise to error except in the most experienced hands. Such a method is unsuitable for a routine laboratory.

In contrast, the tube centrifuging method which has been described gave no discrepancies in 540 observations. It is easy to perform and can be read by inexperienced observers without difficulty. In addition it makes possible the use of antisera which would normally be considered too weak for grouping purposes.

Summary

In 917 consecutive routine determinations of Rhesus groups by the Chown capillary tube method, 20 erroneous results were obtained, nine being false positive and 11 false negative reactions.

These observations were carried out in parallel with the standard tube method, which gave no false results in this series.

False positive Chown tests appear to be due to rouleau formation when suspensions of cells in serum are used : this can be avoided by using saline suspensions.

False negative results can be obtained by the Chown test if insufficient time is allowed for the development of a positive result : factors delaying its appearance are dilution, the use of saline as opposed to serum suspensions, and the use of blood specimens more than five days old.

A method is described for rapid Rhesus group determination in a tube which is incubated for 10 minutes at 37° C. and then centrifuged for five minutes at 2,000 to 2,500 r.p.m. This method gave no discrepancies in 540 observations carried out in parallel with the standard tube method.

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THE ESTIMATION OF DICOUMARIN IN BLOOD

BY

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Dicoumarin (3,3'-methylene-bis (4-hydroxy-coumarin)) is widely used to prolong the blood clotting time in the treatment of thrombosis, dosage being controlled by repeated measurements of the prothrombin index. There are no published data concerning the blood levels of dicoumarin during therapy, nor their correlation with the prothrombin index. The method described in this paper has been devised in order to study this problem.

Dicoumarin is extracted from acidified plasma with ethylene dichloride. This, in turn, is extracted with alkali and the aqueous extract buffered to *p*H 7. It is then coupled with diazotized dianisidine to produce a stable red colour.

Procedure

The following reagents are required.

Ethylene Dichloride.—The technical grade material is washed with 1/5 its volume of normal sodium hydroxide, then with a similar volume of normal sulphuric acid, and finally three times with a similar volume of distilled water.

5N Hydrochloric Acid (Approx.).—Concentrated hydrochloric acid (AR), 50 ml., is diluted with distilled water to 100 ml.

M/15 Disodium Hydrogen Phosphate.—Anhydrous disodium hydrogen phosphate, 9.465 g., is dissolved in distilled water on a water bath, cooled, and made up to 1 litre.

2/15M Potassium Dihydrogen Phosphate in 0.1% Gum Ghatti.—A muslin bag containing 1 g. of gum ghatti "tears" is suspended just below the surface of 500 ml. of distilled water contained in a tall cylinder. After two to three days the gum solution is filtered on a water pump, and 18.145 g. anhydrous potassium dihydrogen phosphate is dissolved in 500 ml. water and made up to 1 litre with the gum solution. The solution is kept in the refrigerator.

Diazo-salt Solution.—Brentamine fast blue salt (Imperial Chemical Industries), 0.25 g., is dissolved in ice-cold distilled water to give 100 ml., and 1 ml. added of normal sulphuric acid. The reagent is mixed and filtered, and kept cold. Kept in the refrigerator, the solution is stable for two weeks.

Standard Dicoumarin Solutions : *Stock Standard* (10 mg./100 ml.).—Pure dicoumarin, 0.1000 g., is dissolved in M/15 disodium hydrogen phosphate to make 1 litre, and stored in a dark bottle in the refrigerator. The solution will keep for at least one month.

Working Standard (1 mg./100 ml.).—The stock is diluted ten times with M/15 disodium hydrogen phosphate. It should be made freshly each time.

Extraction.—A quantity of 5 ml. of plasma or serum (not whole blood) is pipetted into a 1 oz. screw-capped universal container, with a cellophane disk covering the rubber liner

of the cap. Then 1 ml. of 5N hydrochloric acid, and 10 ml. of ethylene dichloride are added and the solution shaken on a Kahn shaker for 30 minutes. It is centrifuged at maximum speed for 15 minutes. Three layers are obtained; an upper aqueous, a lower ethylene dichloride, and a solid middle layer. The upper layer is discarded and the lower layer removed with a teated pipette. About 7 ml. should be obtained. Should an emulsion form in the lower layer, it can be readily broken by brisk stirring with a thin glass rod and recentrifugation. Then 6 ml. of the extract is pipetted into another universal container, 4 ml. of disodium hydrogen phosphate added, and shaken mechanically for five minutes. The solution is centrifuged and the upper layer removed with a teated pipette.

Test.—A 3-ml. quantity of the disodium hydrogen phosphate is measured into a labelled tube. Into the blank tube 3 ml. of M/15 disodium hydrogen phosphate is measured, and into a third tube 3 ml. of working standard. To each is added 1 ml. of potassium dihydrogen phosphate solution, mixed well by tapping, and 0.1 ml. of diazo-reagent, freshly removed from the refrigerator, is added. Mixing is by inversion. The colour is read after ten minutes, preferably with a photo-electric absorptiometer and an Ilford spectrum filter No. 624 (yellow-green) zeroing on the blank. The calculation is made as follows:

$$\frac{4}{3} \times \frac{\text{reading of test}}{\text{reading of standard}} = \text{mg. dicoumarin/100 ml. plasma or serum.}$$

Results.—Normal serum or plasma gives a zero dicoumarin value, but with marked haemolysis; there are apparent values of the order of 0.2 mg. dicoumarin/100 ml. Recovery experiments gave over 95% recovery on every occasion. In patients on prolonged dicoumarin therapy, who had been given over 0.6 g. of dicoumarin, plasma dicoumarin values ranging from 0.9 to 2.8 mg./100 ml. were found, corresponding to prothrombin indices ranging from 50% to 12.5% of normal.

Discussion

Axelrod, Cooper, and Brodie (1949) described a method for estimating dicoumarin by measuring the optical density of an alkaline solution at 315 mμ. n-Heptane was used to extract acidified plasma or urine. They gave no results for human bloods. Their method, although simple, requires quartz cuvettes and a Beckmann type instrument and is hardly suitable for the routine chemical laboratory. Clayton and Larmour (1935) described a coupling reaction for coumarin and melilotic acid, using diazotized p-nitroaniline. However, this reaction lacks specificity and sensitivity and proved unsuitable for dicoumarin. Gömöri (1949) suggested the use of stabilized diazonium salts, readily obtainable as dye-stuff intermediates, for the estimation of phenol. After examining nine such salts, one was found to be suitable at *p*H 7. At a more alkaline reaction a yellow colour was produced, but this was not as good as the red colour for estimation. Further, interference from phenols and salicylates occurred at an alkaline *p*H. There is also a colorimetric method for the estimation of dicoumarin described by Pulver and Kaulla (1948).

The new method will detect dicoumarin in a concentration of 1 μg./ml. of final solution, equivalent to a plasma concentration of 0.13 mg./100 ml. A stabilizing colloid is essential, as the azo-compound responsible for the colour is poorly soluble in water at *p*H 7. Beer's law is obeyed up to a concentration of 15 μg./ml. of final solution. For higher concentrations it is satisfactory to dilute with a *p*H 7 buffer solution.

The reaction seems to have a high degree of specificity. Phenol, tyrosine, and coumarin do not interfere. Salicylates give a similar colour only when their concentration exceeds 20 mg./100 ml.

Summary

Dicoumarin can be coupled with diazotized dianisidine to give a stable red colour. This reaction can be used for the determination of plasma or serum dicoumarin, after extraction with ethylene dichloride.

Dicoumarin concentrations of 0.9 to 2.8 mg./100 ml. have been found in patients on prolonged dicoumarin therapy.

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IMPROVED CULTURE METHODS FOR THE DETECTION OF *PS. PYOCYANEA*

BY

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Williams, Clayton-Cooper, Faulkner, and Thomas (1944) drew attention to the fact that *Pseudomonas pyocyanea* may survive and even multiply in solutions of cetrimide (cetyl-trimethyl-ammonium-bromide). The use of this compound in selective media for the identification of *Ps. pyocyanea* was suggested by Harper and Cawston (1945), who recommended a concentration of 1% in nutrient agar. This medium, however, was shown by Hood (1948) to inhibit the growth of small inocula of *Ps. pyocyanea*. Concentrations of cetrimide small enough to be without this inhibitory action allowed the growth of certain strains of *Proteus vulgaris* and *Bacterium coli*. The value of enriching 1% cetrimide agar with serum or blood was therefore tested, and a medium containing 10% defibrinated horse blood was found satisfactory for dilute inocula of pure cultures and for the examination of swabs from infected burns (Hood, 1948, 1949).

A disadvantage of cetrimide blood agar is its dark-brown colour, which makes it necessary to subculture colonies to a medium (e.g., nutrient agar, or preferably 1% cetrimide agar) on which *Ps. pyocyanea* can be identified by its pigments. In search of a simpler technique we have studied (1) the use of plasma for enrichment; (2) the use of an ultra-violet lamp for the detection of fluorescence on solid media; and (3) the practical value of lower concentrations of cetrimide without enrichment.

Experimental Methods and Results

Enrichment of the Selective Medium with Plasma.—In view of the failure of serum previously reported (Hood, 1948), plasma was chosen for trial as an enrichment because it contains components (fibrinogen and probably other substances) which are present in whole blood but not in serum.

Preparation of the Medium.—Hartley's broth, 900 ml., containing 2% of agar was melted; 10 g. of cetrimide (" cetavlon" I.C.I.) were added, and the mixture was allowed to stand in the steamer for one hour. It was then cooled to 55° C., and 100 ml. of citrated horse plasma were added. The medium was poured in 4-in. petri dishes. Cetrimide horse serum agar and cetrimide horse blood agar used for comparison were prepared in the same way. Human plasma was used in some of the observations recorded. (See footnote to Table I.)

Preliminary Tests.—The growth of *Ps. pyocyanea* on cetrimide plasma agar was tested, using small inocula of 21 strains, 16 of them isolated from infected burns at the Birmingham Accident Hospital, and five kindly supplied to us by Dr. R. Christie, of St. Vincent's Hospital, Melbourne, Australia. Cultures in peptone water were incubated overnight at 37° C., and 0.02-ml. drops of 10^{-5} and 10^{-6} dilutions in 10% broth saline were inoculated in quadruplicate

on the surface of cetrimide plasma agar (C.P.A.) plates. Similar inocula were made on cetrimide serum agar (C.S.A.), on 10% horse blood agar, and (seven strains only) on 1% cetrimide 10% horse blood agar (C.B.A.).

Plates were examined after 24 hours' incubation at 37° C. Colonies on C.P.A. were smaller than those on blood agar (which were sometimes confluent), and somewhat smaller than those on C.B.A. With few exceptions, colonies of *Ps. pyocyanea* on C.S.A. were smaller than those on C.P.A., often so small that the use of serum as an enrichment was not pursued. Viable counts on C.P.A. were not significantly different from counts on C.B.A. and on horse blood agar. Comparison of human and horse plasma showed no significant difference in surface viable counts of five strains tested.

Recognition of Ps. pyocyanea *on Cetrimide Plasma Agar.*—Colonies and confluent growth were recognized by the presence of one or both of the pigments fluorescin and pyocyanin. Confluent growth on C.P.A. almost always showed colour ranging from bluish green to lemon yellow. Separate colonies were often colourless after 24 hours' incubation, but after a further 24 hours most of these were pigmented. Under ultra-violet irradiation (see below) young colonies which were not obviously pigmented often showed characteristic fluorescence.

Suppression of Growth of Bacteria other than Ps. pyocyanea *on C.P.A.*—Gram-negative bacilli, which are less sensitive to the action of cetrimide than other groups of bacteria, were chosen for the tests. Twenty-one strains of *Proteus vulgaris* and 15 strains of *Bact. coli* were heavily inoculated on C.P.A. plates. None of the strains showed any signs of growth after 48 hours' incubation at 37° C.

Detection of Fluorescence by Ultra-violet Irradiation.—A 125-watt Osira H.P. dark glass mercury vapour lamp was enclosed in the upper part of a wooden box with an opening on one side to admit a culture plate for inspection. The box was kept in a corner of the laboratory sheltered from direct daylight. Colonies and confluent growth of *Ps. pyocyanea* on blood agar and on C.P.A. plates held 6 in. below the lamp showed brilliant yellow, green, or greenish-blue fluorescence. No other bacterial species showed yellow or green fluorescence, and the faint blue fluorescence of *Proteus vulgaris* and of some staphylococci on blood agar could usually be distinguished without difficulty. Fluorescence of *Ps. pyocyanea* on C.B.A. was generally poor (Figs. 1 and 2).

Trial of Cetrimide Plasma Agar and Ultra-violet Irradiation.—It was decided to compare cetrimide plasma agar and cetrimide blood agar as media for the isolation of *Ps. pyocyanea* from routine swabs taken at dressings of burns. At the same time the value

TABLE I

COMPARISON OF CETRIMIDE PLASMA AGAR AS A MEDIUM, ULTRA-VIOLET IRRADIATION, AND OTHER METHODS

Medium	*Ps. pyocyanea* Present	Percentage of Total Positives	S.E. of Percentage	Other Organisms Present
*Cetrimide plasma agar	539	84.6	1.43	62
Cetrimide blood agar	552	86.6	1.34	284
Blood agar	311	48.8	1.98	—
Blood agar (with aid of fluorescence) ..	435	68.3	1.84	—
Cooked meat medium	619	97.2	0.65	—

Two thousand and four swabs were examined, and *Ps. pyocyanea* was isolated in 637.

* One thousand six hundred and thirty-four swabs were plated on horse plasma cetrimide agar, and 370 on a medium containing human plasma. Ninety-three of the swabs in the second group yielded *Ps. pyocyanea*, including 67 positive on cetrimide blood agar; the difference between these proportions is not significant.

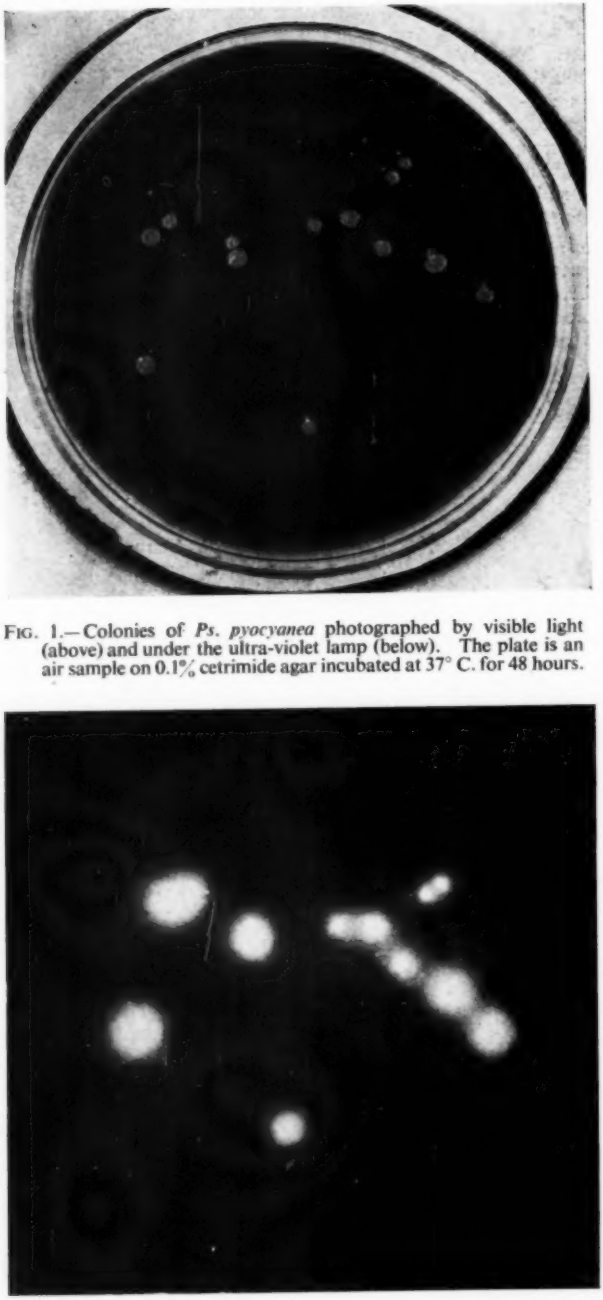


FIG. 1.—Colonies of *Ps. pyocyanea* photographed by visible light (above) and under the ultra-violet lamp (below). The plate is an air sample on 0.1% cetrimide agar incubated at 37° C. for 48 hours.

of inspecting culture plates under the ultra-violet lamp was studied in conjunction with other methods used in the routine bacteriological examination of swabs.

Two thousand and four swabs were inoculated on (1) 10% horse blood agar (containing 6% agar to suppress the swarming of *Proteus vulgaris*); (2) C.B.A.; (3) C.P.A.; (4) Robertson's cooked meat medium.

Blood agar was inoculated first and the cooked meat medium last. Second and third inoculations were made on C.B.A. and C.P.A., each receiving alternate swabs before the other. Twelve swabs were inoculated per plate of each cetrimide medium, without spreading. After overnight incubation at 37° C. blood agar plates were examined by daylight. All colonial types were picked for morphological examination, and Gram-negative bacilli were subcultured to plates of 1% cetrimide agar. Blood agar plates were then examined under the ultra-violet lamp, and fluorescent colonies were picked to 1% cetrimide agar (unless characteristic colonies of *Ps. pyocyanea* had been detected by daylight). Cooked meat cultures and colonies on C.B.A. plates were subcultured to 1% cetrimide agar. C.P.A. plates were examined by daylight; colonies showing no pigment were spread over a small area, and the plates were reincubated for a further 24 hours, after which they and the subcultures from blood agar, C.B.A., and cooked meat medium were examined by daylight and under the ultra-violet lamp.

Growth showing definite green pigment on cetrimide agar or cetrimide plasma agar plates incubated at 37° C. for 24 or 48 hours was called *Ps. pyocyanea*. The morphology of the strains originally isolated from blood agar was checked, and they were all found to be Gram-

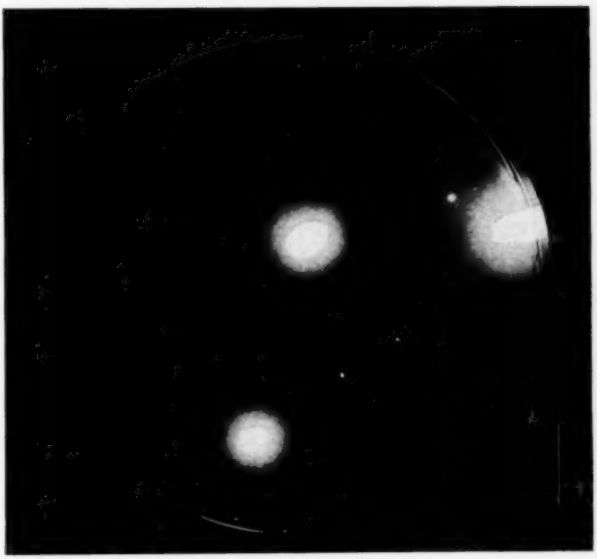
gative bacilli. Biochemical reactions of a number of strains were tested and found to be characteristic. Unpigmented growth picked from 38 cetrimide agar plates, by contrast, gave various biochemical and cultural reactions, none of them consistent with those of *Ps. pyocyanea*.

Table I shows the relative merits of the different media for the isolation of *Ps. pyocyanea*. Although it received the last inoculation, cooked meat medium gave the highest yield of *Ps. pyocyanea* (619 out of 637 positive swabs). Conversely, blood agar, though it was inoculated first, showed a considerably smaller number of cultures positive for *Ps. pyocyanea* (311) than any of the other media. C.B.A. and C.P.A. showed higher proportions of cultures containing *Ps. pyocyanea* than blood agar (552 and 539 respectively), but this difference was reduced when blood agar plates were examined under the ultra-violet lamp as well as by daylight. (Four hundred and thirty-five were positive.)

C.P.A. and C.B.A. received the only strictly comparable inocula, and the difference between the proportions of swabs positive on these media was not significant (difference between percentages = 2.0; S.E. of difference of percentages = 1.96). The difference between the percentages positive on blood agar with and without the aid of the fluorescent lamp was significant (difference between percentages = 19.5; S.E. of difference 2.7).

Practical Value of Lower Concentrations of Cetrimide without Enrichment.—In the course of our studies on the value of plasma as an enrichment it soon became apparent that a rather large proportion of the cultures on C.P.A. which were positive after 48 hours' incubation showed minute colourless colonies and sometimes no recogniz-

FIG. 2.—Young colonies of *Ps. pyocyanea* photographed under the ultra-violet lamp. The plate (0.1% cetrimide agar) had been incubated 24 hours at 37° C. Photograph below shows 24 hours' confluent growth on 0.1% cetrimide agar of subcultures from cooked meat medium, under the ultra-violet lamp.



able growth on the day after inoculation. Another fact which came to light (Table I) was the presence of coliform bacilli other than *Ps. pyocyanea* on many plates of C.B.A. (Two hundred and eighty-four cultures on C.B.A. as against 62 cultures on C.P.A. showed the presence of miscellaneous coliforms.) In spite of this, however, C.B.A. was found to be a valuable selective medium, and the objections previously raised against the use of lower concentrations of cetrimide were therefore brought into question. A pale medium less inhibitory to *Ps. pyocyanea* than C.P.A. and no less selective than C.B.A. would probably be superior to either. These considerations prompted a study on the merits of unenriched cetrimide agar containing reduced concentrations of cetrimide.

As a preliminary experiment a range of doubling dilutions of cetrimide in agar from 0.03% to 1.0% was inoculated with 22 strains of *Proteus vulgaris* and 14 strains of *Bact. coli*. Six of these strains grew (three of them poorly) on 0.12% cetrimide agar. Fifteen strains grew on a medium containing 0.06% cetrimide. By comparison, 18 of the *Proteus* and *Bact. coli* strains grew on C.B.A. inoculated at the same time. Swarming occurred in some strains on 0.03% cetrimide agar, but was not observed in the presence of higher concentrations of cetrimide. Drop counts of 12 strains of *Ps. pyocyanea* were done on the same range of media and on C.P.A. Growth on 0.06% and 0.12% cetrimide agar was richer in quality and in colonial counts than growth on C.P.A. In this test a few strains showed evidence of slight inhibition by cetrimide at all concentrations, including 0.03% which allowed *Proteus vulgaris* to swarm. As C.P.A. had already shown its value as a selective medium, however, it was decided that a trial of 0.1% cetrimide agar for the isolation of *Ps. pyocyanea* from burns swabs was indicated.

Trial of 0.1% Cetrimide Agar

One thousand and fifty-nine swabs from burns were inoculated on 10% horse blood agar (with 6% agar), on 0.1% cetrimide agar (C.A.), on C.P.A., and on cooked meat medium. Cetrimide agar and C.P.A. were made comparable by reversing the order of inoculation of successive swabs on the two media.

Examination of the cultures was done in the way described above for the study of plasma as an enrichment. In addition, plates of C.A. and C.P.A. were examined under the ultra-violet lamp for fluorescence after 24 hours' and 48 hours' incubation.

Table II shows the relative merits of C.A. and C.P.A. Two hundred and eighty-four swabs were found to be positive on C.A. compared with 250 which were positive on C.P.A., indicating a slight but significant advantage in the use of C.A. (difference between the

TABLE II

COMPARISON OF 0.1% CETRIMIDE AGAR WITH 1.0% CETRIMIDE PLASMA AGAR

		Growth (Hours)			Appearance of Pigment (Hours)			Appearance of Fluorescence (Hours)		
		24	48	Total	24	48	Total	24	48	Total
0.1% cetrimide agar	Positive ..	271.0	13.0	284.0	167.0	113.0	280.0	231.0	28.0	259.0
	% of total positives	77.2	3.7	80.9	47.6	32.2	79.8	65.8	7.9	73.7
	S.E. of % ..	2.25	—	2.1	2.66	2.49	2.14	2.53	1.47	2.35
1.0% cetrimide plasma agar	Positive ..	199.0	51.0	250.0	113.0	127.0	240.0	166.0	51.0	217.0
	% of total positives ..	56.7	14.5	71.2	32.2	36.2	68.4	47.3	14.5	61.8
	S.E. of % ..	2.63	1.87	2.4	2.49	2.57	2.49	2.66	1.87	2.59

One thousand and fifty-nine swabs were examined and *Ps. pyocyanea* isolated in 351.

percentages = 9.7; S.E. of difference = 3.2). When the proportions of cultures on the two media which could be identified by pigment after 24 hours' incubation were compared, the superiority of C.A. was more definite (difference between percentages showing pigment after 24 hours = 15.4; S.E. of difference = 3.65).

The superiority of the cetrimide media to blood agar (197 positives), and their inferiority to cooked meat medium (333 positives), were again demonstrated in this trial.

The practice of examining cetrimide plates by ultra-violet radiation was vindicated. After 24 hours' incubation, 231 cultures on C.A. showed fluorescence, while only 167 showed definite pigment; on C.P.A., 166 cultures were fluorescent after 24 hours' incubation, compared with 113 which were pigmented.

Pigment Production on 0.1% *Cetrimide Agar.*—In spite of the greater frequency on C.A. than on C.P.A. of pigmented colonies after 24 hours' incubation, confluent growth on the latter medium was generally found to give better pigment. By the use of a glass electrode it was shown that 1% cetrimide in distilled water had a *p*H of 7.75, compared with 0.1% cetrimide in distilled water, which had a *p*H of 6·98. It is known that pyocyanin production is inhibited by an acid reaction, in which a red pigment is produced (Wrede, 1930; Young, 1947). Red pigment was observed in some of the cultures on 0.1% cetrimide agar and occasionally on C.P.A. cultures.

Parallel tests on 0.1% cetrimide agar prepared without adjusting the *p*H and on the same medium adjusted to *p*H 7.8 and *p*H 8.0 were done, using a number of strains of *Ps. pyocyanea.* Both green pigmentation and fluorescence were more pronounced at *p*H 7.8 and *p*H 8.0 than at *p*H 7.0. As the medium at *p*H 8.0 was slightly inhibitory to some strains of *Ps. pyocyanea*, *p*H 7.8 was considered the best reaction for the medium.

Discussion

The most satisfactory of the selective media under study was arrived at in three stages. First, 1% cetrimide plasma agar was shown to compare favourably with 1% cetrimide blood agar in the yield of *Ps. pyocyanea* from burns swabs plated on the two media; as it was unnecessary to subculture the growth from cetrimide plasma agar for the demonstration of pigment, this medium was adopted for routine purposes. In the second stage, 0.1% cetrimide agar without enrichment was compared with 1% cetrimide plasma agar in a similar trial on swabs from burns, and a slight, but significant, advantage was scored by the unenriched 0.1% cetrimide agar, especially in the yield of positive results after 24 hours' incubation. The advantage of this medium was, however, offset by the poor quality of pigment in confluent growth of *Ps. pyocyanea.* Adjustment of the medium to *p*H 7.8 was the third stage, which showed that growth on 0.1% cetrimide agar could be as green as that on cetrimide plasma agar. As the former medium was more sensitive and apparently no less selective than the latter, it is considered the most satisfactory of the cetrimide media examined.

A combination of methods, such as those described in this paper, is undoubtedly superior to any single method of culture. Fluid media provide the best opportunity for isolating the organism from very small inocula, but they give no indication of the load of infection. Cetrimide media, and particularly 0.1% cetrimide agar, have shown their superiority to horse blood agar for direct plating of swabs, but may inhibit small inocula of the occasional sensitive strain of *Ps. pyocyanea*; such strains, however, would almost certainly grow on subculture of the fluid medium inoculated from the same swab. A blood agar plate examined by visible light and under a

fluorescent lamp provides a quicker result in some cases than either of the selective media studied. As it is possible by this method to recognize *Strep. pyogenes*, *Staph. aureus*, *Ps. pyocyanea*, and other aerobic flora on the same culture plate, it may be recommended for routine purposes when pressure of work precludes the use of more sensitive techniques. Ultra-violet irradiation of blood agar subcultures from liquid media often shows good fluorescence, but this may be obscured by the blue fluorescence of *Proteus vulgaris* which often accompanies *Ps. pyocyanea* in burns.

Summary

Three solid media containing cetrimide were compared with each other and with non-selective methods for the isolation of *Ps. pyocyanea* from burns swabs. Cetrimide agar, 0.1%, adjusted to *p*H 7.8 was found to be the most suitable of the selective media. Subculture to a selective medium from cooked meat broth was the most effective method for scanty inocula.

Examination of growth on solid media for fluorescence under an ultra-violet lamp with a dark glass filter was found useful as an aid to the recognition of *Ps. pyocyanea*.

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THE EFFECTS OF HEAT AND SODIUM ETHYL MERCURI-THIO-SALICYLATE ON THE STAPHYLOCOAGULASE TEST

BY

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The ability of certain strains of staphylococci to clot plasma has been known since 1903 when it was first described by Loeb working with goose plasma. The value of this observation and its application to routine bacteriology did not become apparent until Chapman, Berens, Peters, and Curcio (1934) and Chapman, Berens, Nilson, and Curcio (1938) showed that coagulase was related to pathogenicity. Cruickshank (1937), investigating staphylocoagulase, showed that pathogenicity to rabbits and the production of coagulase were closely related.

Cadness-Graves, Williams, Harper, and Miles in 1943 described a slide test for the detection of coagulase-producing staphylococci, and showed that this method was closely correlated with the tube method used hitherto. Indeed, these two tests parallel one another so closely that they are commonly assumed to be manifestations of the same reaction.

While engaged in routine testing of staphylococci for their coagulase production, we found that, while the slide results were in accordance with the clinical diagnosis, the results of the tube test were consistently negative. At this time we were using a batch of American Army freeze-dried product as the source of our plasma. This discrepancy led us to the following investigations.

Methods

Tube Method.—An 18-hour broth culture of the staphylococcus, 0.1 ml., was added to 0.5 ml. of citrated human plasma diluted 1 : 10 with sterile normal saline in 4 in. × $\frac{3}{8}$ in. tubes. This mixture was then incubated for two hours at 37° C. in a water bath. A control consisting of the same plasma plus 0.1 ml. of sterile broth was set up in each group of tests.

Slide Method.—An 18-hour culture of the staphylococcus grown on plates of plain nutrient agar was used in the slide test. Two loopfuls of distilled water were placed on a clean slide and a sufficient amount of a single colony was emulsified to produce a homogeneous turbidity. A loopful of undiluted plasma was placed beside the staphylococcal suspension and the two mixed by rocking. With this technique nearly all positive strains give immediate clumping. Some doubtful and weak reactions required stirring with a loop to confirm the result.

Reconstituted plasma was made up in a concentration of 5 g. of the dried product in 100 ml. of sterile distilled water to give a plasma of normal concentration. The staphylococci used were freshly isolated.

Results

The following results were obtained with reconstituted American freeze-dried plasma and with fresh citrated human plasma when tested against 63 strains of staphylococci (Table I).

TABLE I

COMPARISON OF FRESH AND RECONSTITUTED PLASMA IN COAGULASE TESTING OF 63 STRAINS OF STAPHYLOCOCCI

Total No. Staph. Strains Tested	Staphylococcal Strains Tested	Tube Method		Slide Method	
		Fresh Plasma	Reconstituted Plasma	Fresh Plasma	Reconstituted Plasma
63	No. positive	48	0	48	48
	No. negative	15	63	15	15

As the slide test depends for its effect on the formation of a fibrin web which contracts and so clumps the staphylococci together, these results show that the reconstituted plasma still contains some of its original fibrinogen.

Thirty-seven strains of staphylococci were tested using dried plasma from the National Blood Transfusion Service. Complete correlation in both tube and slide tests was obtained when compared with fresh citrated human plasma.

The results of laboratory freeze drying were next observed; 2-ml. samples of freeze-dried plasma were reconstituted at varying times after processing, and then tested with 10 strains of staphylococci. The first sample was reconstituted immediately after preparation, the rest at weekly intervals up to six weeks after freeze-drying. From this it emerged that laboratory freeze-drying of plasma followed by storage at room temperature had no detectable effect on the reactions with staphylocoagulase within this time.

American freeze-dried plasma contains merthiolate (sodium ethyl mercuri-thiosalicylate) 1/50,000 as a preservative, and the effect of this substance on the coagulase test was therefore investigated.

Fresh citrated human plasma was treated with merthiolate to give final concentrations of 1/10,000 and 1/100,000, the mixture being allowed to stand for 24 hours at 4° C. At a concentration of 1 in 100,000, merthiolate was found to have a retarding effect on the tube coagulase test which varied with the different strains of staphylococci as shown in Table II, whereas the slide test with plasma containing 1/100,000 merthiolate showed complete correlation with the slide test using normal fresh plasma.

The effect of merthiolate at 1/10,000 was next investigated. In this experiment none of the 16 positive strains of staphylococci clotted the plasma in the tube in two hours. Therefore a further reading was taken at 18 hours. Despite the

TABLE II

COMPARISON OF NORMAL PLASMA AND NORMAL PLASMA CONTAINING MERTHIOLATE 1 IN 100,000 (TUBE TEST)

Total Number of Strains Tested	16	
	Normal Plasma	Plasma + Merthiolate
Number of strains coagulase positive in 30 min.* ..	15	0
Number of strains coagulase positive in 60 min. ..	16	4
Number of strains coagulase positive in 90 min. ..	16	10
Number of strains coagulase positive in 120 min. ..	16	14

* Readings taken at 30-min. intervals up to 2 hours.

increased time the results were again negative. With normal fresh plasma all the positive strains gave a coagulum in 30 minutes.

In view of the fact that 24 hours' exposure to 1/10,000 merthiolate at 4° C. abolished the tube coagulase test, further experiments were undertaken with that sample of plasma to ascertain the effects of time of exposure to merthiolate on this phenomenon. The results in Table III show that the duration of contact between plasma and merthiolate has a direct relationship to the tube coagulation test.

TABLE III

EFFECT OF DURATION OF CONTACT BETWEEN NORMAL PLASMA AND MERTHIOLATE (1 : 10,000)

	Normal Plasma	Duration of Contact between Plasma and Merthiolate				
		1 hour	2 hours	4 hours	12 hours	24 hours
Number coagulase positive	16	8*	5†	2‡	0	0
Number coagulase negative	0	8	11	14	16	16

* Four of these strains were positive after 90 min. and 4 after 120 min. incubation at 37° C. † One strain positive after 90 min., 4 after 120 min. incubation. ‡ Both positive after 120 min. incubation.

The possible mode of action of merthiolate was then studied. The ability of merthiolated plasma to clot in the presence of calcium ions and thrombokinase was confirmed.

The clotting time of normal citrated plasma was 18 seconds. This plasma was then treated with merthiolate to give a final concentration of 1/10,000. The mixture was left for 24 hours at 4° C.; then its clotting time was estimated after the addition of calcium ions and thrombokinase, and found to be 21 seconds (average of three readings).

From Table I it will be seen that all the tube coagulase tests were negative whereas the slide results were normal using American reconstituted plasma. An investigation was carried out to determine if other methods would abolish the tube test while leaving the slide test unaffected. With this end in view the effect of heat on the tube and slide coagulase test was investigated.

Human plasma was taken from volunteers, immediately heated for varying periods at 56° C., and then used in both slide and tube tests. Each batch of heated

plasma was tested with strains of staphylococci. As the results in Table IV show, within four minutes of heating at 56° C. the tube test was rendered completely negative while the slide test remained positive. It was noted, however, that the slide

TABLE IV

EFFECT OF HEATED PLASMA ON TUBE AND SLIDE COAGULASE TESTING OF 20 STAPHYLOCOCCAL STRAINS

	Coagulase	Normal Plasma	Heating Time (Min.)						
			1	2	4	8	15	30	60
Tube ..	Positive	16	16	6	0	0	0	0	0
	Negative	4	4	14	20	20	20	20	20
Slide ..	Positive	16	16	16	16	16	16	16	16
	Negative	4	4	4	4	4	4	4	4

test, while positive in plasma heated for 30 and 60 minutes, showed a weaker coagulum than in the control.

The tests were repeated using the same dilution of plasma (1 : 10 in the slide test as in the tube test) to investigate the possibility that the different concentrations of plasma in the usual tube and slide tests may have played some part in the results shown in Table IV. However, as is shown in Table V, the diluted plasma behaved in the same way as the undiluted.

TABLE V

EFFECT OF HEATING DILUTED PLASMA ON TUBE AND SLIDE COAGULASE TESTING OF 10 STAPHYLOCOCCAL STRAINS

	Coagulase	Normal Plasma Diluted 1 : 10	Heating Time (Min.)						
			1	2	4	8	15	30	60
Tube ..	Positive	7	7	4	1	0	0	0	0
	Negative	3	3	6	9	10	10	10	10
Slide ..	Positive	7	7	7	7	7	7	7	7
	Negative	3	3	3	3	3	3	3	3

Discussion

The effect of heating and merthiolate in selectively destroying the tube coagulase activity of plasma while affecting the slide coagulase reaction much less or not at all has been demonstrated.

Wilson Smith and Hale (1944), investigating the mechanism of the tube coagulase test, showed that the plasma contributes a factor which enables staphylocoagulase to convert fibrinogen into fibrin. This factor is known as the activator substance and is heat labile.

The mechanism of the slide coagulase test was investigated by Cadness-Graves *et al.*, who showed that purified fibrinogen would work very well in the slide test in the place of plasma.

Both merthiolate and heating to 56° C. modify human plasma in such a way that a tube coagulase test is invariably negative whereas the plasma still gives positive reactions when employed in the slide test. This effect suggests that there is some difference in the underlying mechanism of the two tests. The lability of the coagulase reacting factor in plasma suggests that it might be at this point in the coagulase mechanism that heat and merthiolate act.

Summary

Reconstituted plasma without a preservative is a satisfactory source of plasma for the tube and slide coagulase tests.

Reconstituted plasma with merthiolate as a preservative or fresh heated plasma does not provide a satisfactory plasma for the tube coagulase test, but gives good results in the slide test.

We wish to thank Dr. Cadness-Graves for advice and assistance with the references.

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THE EXCRETION OF PREGNANEDIOL AND 17-KETOSTEROIDS DURING THE MENSTRUAL CYCLE IN BENIGN HIRSUTISM

BY

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In 1949 Koets reported a variation in the urinary 17-ketosteroid excretion occurring during the menstrual cycle of women with benign hirsutism, the maximal excretion being at, or about, the presumed time of ovulation.

It has been established that in normal women there is little or no cyclical fluctuation in the excretion of 17-ketosteroids (Werner, 1941; Patterson, McPhee, and Greenwood, 1942; Venning and Kazmin, 1946), although Furuhjelm (1948), from a careful study of 19 healthy subjects, concluded that there is a tendency for excretion to decrease during menstruation and to rise slightly during the secretory phase.

Although the condition of benign hirsutism is often accompanied by some menstrual irregularity, Koets only assumed, but did not establish, that his patients had ovulated. It seemed of some importance to define the relationship of the peak of 17-ketosteroid excretion, which he described, to events in the ovarian cycle.

Venning and Browne (1937) found that the rate of excretion of pregnanediol during the menstrual cycle could be related to the formation and regression of the corpus luteum. Furthermore, there is now no doubt that pregnanediol is one of the principal metabolites of progesterone (Buxton and Westphal, 1939; Hamblen, Cuyler, and Hirst, 1940; Venning and Browne, 1938, 1940; Heard, Bauld, and Hoffman, 1941; Sommerville and Marrian, 1950). It was decided, therefore, to estimate the 17-ketosteroid and pregnanediol concentrations of each specimen of urine collected during the menstrual cycles of a number of hirsute women. It is the purpose of this paper to report the results of these experiments.

Material of the Investigation

Twenty-four-hour specimens of urine were collected from four healthy young women, aged 22 to 29 years, with a normal menstrual history and normal distribution and quantity of body hair, and from ten patients who had been referred to the Endocrine Clinic at Guy's Hospital complaining of hirsutism, or in whom hirsutism had been noted in the course of examination for some other complaint. Urine collections were made between November and May on the seventh, 14th, 21st and, where possible, between the 25th and the 28th days after the onset of the last menstrual period; the date of the collection of the last specimen was adjusted to suit

TABLE I

CLINICAL FEATURES OF THE ABORTIONAL GROUP

Case Number	Age	Age at Onset	Colour of Hair	Complexion	Greasy Skin	Married (M) or Single (S) Children (C)	Distribution of Excess Hair								Age at Menarche	Present Menstrual State	Other Observations
							Upper Lip	Beard Area	Shoulders	Breasts	Abdomen	Back	Thighs	Legs			
1	19	17½	Dark	V. pale	No	S	–	+	+	++	+	–	+	–	13	Irregular roughly $\frac{7}{28–33}$	Jewish grandparents
2	21	14	Fair	Fair	Yes Acne	S	–	+	+	+	+	+	–	–	14	Regular $\frac{3–4}{28}$	Slightly obese
3	21	18	Fair	Fair	Yes	S	+	+	–	–	+	–	+	+	11	Regular $\frac{2}{23}$	
4	22	18	Dark	Fair	No	S	+	+	–	+	+	+	+	+	11	Irregular $\frac{5–6}{23–28}$	Obese. Mother Italian
5	27	17	Dark	Dark	No	M C2	+	+	–	–	–	–	+	–	13	Fairly regular $\frac{6}{26–30}$	
6	27	25	Dark	Dark	Yes	S	+	+	–	+	+	+	+	–	13 or 14	$\frac{4–5}{30}$ Scanty loss	Onset coincided with emotional upset
7	27	17	Dark	Pale	Yes	M	–	Slight	–	+	+	+	++	+	13	$\frac{3–4}{28–29}$	Onset coincided with emotional upset
8	27	26	Dark	Dark	Yes	M C2	+	+	–	–	–	–	–	–	13	$\frac{6}{28}$ but now has amenorrhoea	Severe emotional upset. Unhappy home life
9	29	12	Fair	Sallow	Yes Acne	M	+++	+	+	+	+	+	+	+	13½	Irregular $\frac{3}{28–34}$	Consulted on account of sterility. M 18/12
10	31	29	Dark	Ruddy	Yes	M C2	+	++	–	+	+	–	–	+	10	$\frac{2½–3}{21}$ V. heavy loss	Unhappy home life. Many other symptoms

individual menstrual irregularities as required. Both the normal subjects and the patients carried on their daily work as usual during the period of the experiment.

The clinical features of the abnormal group are summarized in Table I. In no case was there a family history of hirsutism. It seems worth pointing out that the onset of the hirsutism in four cases (Nos. 6, 7, 8, and 10) dated from the occurrence of severe emotional upset and personal unhappiness.

Methods

Urine specimens were collected in bottles containing 3 g. of a mixture of powdered thymol and salicylic acid (2 : 1 w/w) to prevent bacterial growth (Bisset, Brooksbank, and Haslewood, 1948). Estimations were made within 24 hours of the completion of the collection of urine wherever possible, and never more than 48 hours after the collection was finished. Where any delay occurred, specimens were stored at 2–4° C. until they were processed.

Pregnanediol was estimated by the method of Sommerville, Gough, and Marrian (1948).

Extracts of the urine containing the neutral ketonic fraction were prepared by the method of Talbot, Butler, and MacLachlan (1940), modified only as regards the preparation of the crude neutral fraction, which was carried out as follows:

Urine, 250 ml., and sulphur-free toluene, 50 ml., were placed in a flask of 500 ml. capacity which was attached by a ground glass joint to a bulb reflux condenser. The urine was brought to the boil on an electric hot-plate and 25 ml. concentrated hydrochloric acid added down the condenser. Boiling was continued for exactly 10 minutes. The apparatus was then disconnected and the flask and contents cooled rapidly in running water.

The urine-toluene mixture was transferred to a separating funnel of 1,000 ml. capacity and shaken. Separation of the emulsion was aided by filtration through a Whatman No. 1 filter paper on a Buchner funnel using gentle suction. The urine which separated from the emulsion was added to the urine pool before each subsequent extraction. The urine was extracted twice more with 50 ml. toluene, separation of the emulsion being similarly effected.

The combined toluene extracts were washed three times with 50 ml. 10% (w/v) sodium hydroxide solution, and twice with 50 ml. distilled water.

The extract was then dried with anhydrous sodium sulphate and the toluene evaporated on a boiling water bath at a pressure of 30 mm. of mercury.

The colorimetric assay of the neutral ketonic fraction was performed by the method of Holtorff and Koch (1940), except that the dilution of the final coloured solution was made with 70% ethanol instead of 95% as used by these authors.

Creatinine was estimated by the method of Folin (1905a).

Results

Table II shows the results of estimations of pregnanediol, 17-ketosteroids, and creatinine on the urines of four healthy subjects.

The creatinine excretion was estimated in order to exclude incompleteness of urine collections. Wang (1939) and Albanese and Wangerin (1944) have shown that the creatinine excretion in any individual is not so constant as was originally assumed by Folin (1905b), and that fluctuations of the order of 10 to 25% are the rule. (Figures in parentheses in Tables II and III refer to the concentration of steroids in mg. per g. of creatinine). This method of correcting concentrations of steroids in 24-hour urine specimens to an approximately constant volume of urine for the individual was proposed by Jayle and Libert (1946), and seems acceptable provided that there is no gross diuresis and the limitations of using the creatinine excretion as a yardstick are borne in mind.

TABLE II

EXCRETION OF PREGNANEDIOL, 17-KETOSTEROIDS, AND CREATININE DURING MENSTRUAL CYCLES OF FOUR HEALTHY WOMEN WITH NORMAL HAIR DISTRIBUTION

Subject No.*	Day of Cycle	Pregnanediol (mg. per 24 hrs.)	17-Ketosteroids (mg. per 24 hrs.)	Creatinine (g. per 24 hrs.)	Urine Volume (ml. per 24 hrs.)
1	7	0.28 (0.35)†	8.0 (10.2)	0.79	625
	14	0.44 (0.35)	11.2 (9.0)	1.25	980
	20	2.20 (2.09)	12.9 (12.3)	1.05	880
2	7	0.81 (0.55)	5.3 (3.6)	1.48	1650
	14	0.43 (0.33)	5.8 (4.4)	1.33	1460
	21	2.35 (1.87)	6.9 (5.5)	1.26	1580
	27	1.07 (0.81)	7.5 (5.6)	1.33	1370
3	7	0.82 (0.79)	7.2 (6.9)	1.04	1120
	14	1.71 (1.31)	9.9 (7.6)	1.31	1550
	21	0.81 (0.64)	8.1 (6.4)	1.26	1550
4	7	0.91 (0.83)	4.7 (4.3)	1.10	1100
	14	0.51 (0.43)	8.0 (6.7)	1.20	1240
	21	0.53 (0.52)	7.5 (7.3)	1.02	890
	27	0.91 (0.76)	6.4 (5.3)	1.21	1200

*Subjects 1 and 3 menstruated on the 21st and 22nd days respectively, Subjects 2 and 4 on the 28th day.

†Denote the concentrations of steroids excreted expressed in mg. per g. creatinine.

Reference to Table II shows that Subjects 1, 2, and 3 exhibited increased luteal activity towards the end of the cycle, as shown by the rise in pregnanediol excretion to more than 1.5 mg. a day. Venning and Browne (1937) and Cope (1940) showed that the excretion of pregnanediol in the luteal phase of the menstrual cycle varied from 1 to 8 mg. of the conjugated excretory product, pregnanediol glucuronide, daily; during the follicular phase only minimal quantities are usually excreted. Case 4 failed to show any real peak of pregnanediol excretion; it is possible that her peak excretion was missed through doing the estimations at weekly intervals, but experience of doing the estimations in this way suggests that this was unlikely, and that she did not, in fact, show any increase of luteal activity and therefore did not ovulate (Venning and Browne, 1937).

The 17-ketosteroid excretion of all these subjects varied during the cycle by a maximum of 3.3 mg. (Subject 4), the greater fluctuation found in Subject 1 being almost certainly attributable to the fact that the first urine specimen was incomplete. There is a tendency in all subjects for the 17-ketosteroid excretion to increase towards the middle or end of the cycle.

Table III shows the results of estimations of pregnanediol, 17-ketosteroids, and creatinine on the urines of ten women with hirsutism.

Case 9 did not show any luteal activity, as revealed by a rise in pregnanediol excretion during her cycle. She is therefore comparable with Subject 4 of the healthy series (Table II).

Case 8, who had had amenorrhoea for 13 months before the experiment and during it, consistently excreted amounts of pregnanediol which might normally be expected

TABLE III

EXCRETION OF PREGNANEDIOL, 17-KETOSTEROIDS, AND CREATININE AT INTERVALS DURING THE MENSTRUAL CYCLES OF TEN WOMEN WITH HIRSUTISM

Case No.	Day of Cycle	Pregnanediol (mg. per 24 hrs.)	17-Ketosteroids (mg. per 24 hrs.)	Creatinine (g. per 24 hrs.)	Urine Volume (ml. per 24 hrs.)
1	7	0.58 (0.36)*	9.8 (6.13)	1.60	1440
	14	0.56 (0.35)	13.8 (8.7)	1.58	1450
	20	1.16 (0.68)	13.5 (7.9)	1.70	1920
	24	0.73 (0.56)	13.7 (10.5)	1.30	1125
	32	2.31 (2.05)	14.6 (12.9)	1.13	1040
2	7	3.10 (1.64)	15.2 (8.0)	1.89	1670
	14	2.85 (1.59)	25.8 (14.3)	1.80	1650
	21	6.65 (3.80)	28.7 (16.4)	1.75	1538
	27	2.10 (1.31)	18.7 (11.3)	1.00	900
3	7	0.54 (0.38)	15.2 (10.7)	1.42	1450
	15	0.94 (0.99)	14.4 (15.2)	0.94	700
	21	1.69 (0.92)	18.5 (10.1)	1.84	1740
4	7	0.62 (0.39)	11.0 (6.9)	1.60	1700
	15	0.93 (0.67)	16.5 (11.8)	1.40	1320
	22	5.15 (3.43)	21.7 (14.5)	1.50	1445
5	7	0.91 (0.57)	12.8 (8.0)	1.58	1405
	14	0.89 (0.62)	12.6 (8.7)	1.45	1070
	21	5.73 (3.58)	33.0 (20.6)	1.63	1755
	27	1.33 (0.84)	13.2 (8.2)	1.59	1410
6	7	0.79 (0.36)	13.1 (5.9)	2.20	2365
	14	2.05 (0.95)	33.6 (15.5)	2.17	2210
	21	1.46 (1.47	11.9 (12.1)	0.99	820
7	7	0.45 (0.30)	14.2 (9.5)	1.49	1365
	14	0.93 (0.72)	14.7 (11.3)	1.30	1250
	21	3.60 (3.53)	16.1 (15.8)	1.02	1000
	26	1.90 (1.26)	11.3 (7.5)	1.51	1450
8	7	4.12 (1.82)	45.4 (20.0)	2.27	2520
	16	2.23 (1.01)	46.8 (22.3)	2.11	2810
	21	2.35 (1.68)	23.3 (16.5)	1.40	1400
	28	5.65 (2.29)	54.2 (22.0)	2.46	2490
9	7	0.54 (0.43)	9.9 (7.7)	1.28	1460
	14	0.14 (0.10)	8.8 (6.6)	1.32	1538
	21	0.54 (0.39)	6.2 (4.5)	1.40	1730
	27	0.09 (0.06)	6.5 (4.7)	1.38	1725
10	7	1.26 (0.78)	9.8 (5.8)	1.62	2090
	14	0.50 (0.27)	12.5 (6.8)	1.83	1630
	21	1.88 (1.14)	16.7 (10.1)	1.65	1740
	27	0.73 (0.45)	14.4 (9.0)	1.60	1600

Case 1 menstruated on the 35th day; case 3 on the 22nd day; case 4 on the 24th day; case 6 on the evening of the 21st day; case 8 had amenorrhoea. All the other cases menstruated on the 28th or 29th days.

Sample 2 from case 3 is known to be incomplete. Sample 3 from case 6 consists of day urine only, since she began to menstruate at 6 p.m. of the 21st day. Sample 3 from case 7 is known to be incomplete by about 7 hours during the day.

*Refer to the concentrations of steroids excreted expressed in mg. per g. creatinine.

during the luteal phase of the cycle. This finding was associated with a raised 17-ketosteroid excretion throughout. It is possible that the colour developed by pregnanediol in the estimations may have been enhanced by the presence of abnormal amounts of 17-ketosteroids, thus giving a falsely high result (Sommerville *et al.*, 1948). On the other hand, if the pregnanediol results are true ones, it supports the possibility that this is a case of suprarenal hyperplasia rather than benign hirsutism, since ovarian tumour was excluded at clinical examination.

It is doubtful whether Case 3 can be regarded as having shown any luteal activity. The second urine collection from this patient was almost certainly incomplete, and there is reason to believe that the third specimen was collected over a period of more than 24 hours. The results of pregnanediol estimations imply that no definite peak of excretion occurred.

The remaining cases all excreted an increased amount of pregnanediol towards the end of their cycles, the quantity varying from 1.88 mg. by Case 10 to 6.65 mg. by Case 2. Case 2 is of additional interest, since the levels of pregnanediol excretion during the follicular phase were higher than is usual.

The 17-ketosteroid excretion of these cases, in which the results of the pregnanediol estimations indicate ovulation, show a marked cyclical fluctuation. This is most pronounced in Cases 2, 4, 5, and 6, and less so in Cases 1 and 10. There is little doubt that it would have been more significant in Case 7 if the third urine collection had not been incomplete. These findings contrast with those of the normal subjects and with those of Case 9. She did not apparently ovulate, and her 17-ketosteroid excretion decreased slightly throughout the month.

The peak of 17-ketosteroid excretion seems to coincide with that of pregnanediol, a fact emphasized by the results in Case 9, where no peak of pregnanediol excretion was detected, and no cyclical variation of 17-ketosteroid excretion occurred.

Discussion

These results confirm Koets's (1949) findings of a variation in 17-ketosteroid excretion occurring during the menstrual cycle of hirsute women who ovulate. However, instead of the peak 17-ketosteroid excretion occurring at the time of ovulation, as Koets asserted, the results presented here relate it closely to luteal activity and the excretion of pregnanediol. It is possible that this association may be quite fortuitous, the increased excretion of 17-ketosteroids being attributable directly to ovarian activity, since Parkes (1937) and Deanesly (1938) have produced some evidence for ovarian secretion of androgenic material, which as yet remains unidentified: or the suprarenal cortex may be stimulated to increased 17-ketosteroid production by some substance produced in the ovary or elsewhere at this time in the cycle. The latter possibility is being investigated and the results will be published later. It is enough to say that so far there is no indication of any increase of suprarenal activity with respect to the excretion of other steroids at the time when 17-ketosteroid excretion is maximal.

It may be that the substance participating in the Zimmerman reaction, and thus contributing to the colour formed by 17-ketosteroids in the urine extracts, is not itself a 17-ketosteroid but a 20-ketosteroid such as pregnanolone, which is known to be an intermediate metabolite of progesterone.

If the excretion of pregnanediol and of 17-ketosteroids in benign hirsutism is related, the possibility that this points to an abnormality of progesterone metabolism cannot be ignored. Marrian (1939) suggested a possible metabolic pathway for the conversion of progesterone to C19 17-ketosteroids, but it has not so far proved possible to establish by isolation studies that such a process occurs, in spite of the strong theoretical probability. It is conceivable that in the hirsute woman there is an exaggeration of normal progesterone metabolism along these lines. Preliminary observations on this aspect of the problem have been started in this Department, but it is too early to draw any definite conclusions yet.

Summary

Pregnanediol and 17-ketosteroid estimations were made at intervals during the menstrual cycles of four healthy women and ten women with hirsutism.

In contrast to the healthy group the women with hirsutism who ovulated showed a distinct cyclical fluctuation of 17-ketosteroid excretion, which was apparently related to the peak of pregnanediol excretion occurring during the luteal phase of the cycle. One hirsute woman, who did not show any peak of pregnanediol excretion, showed no increase of 17-ketosteroid excretion.

The results are discussed, and further steps in the work indicated.

I am indebted to Dr. P. M. F. Bishop for allowing me to carry out these experiments on his patients and for his advice and interest. Dr. R. M. de Mowbray kindly arranged for patients to see me.

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KING-ARMSTRONG PHOSPHATASE ESTIMATION BY THE DETERMINATION OF LIBERATED PHOSPHATE*

BY

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As a result of many requests for a phosphatase method which would use the phenyl phosphate substrate of King and Armstrong (1934), but which would substitute a determination of the liberated inorganic phosphate for that of phenol, we have developed two procedures which are described in this paper. The principal reason for a method in which the final measurement is of inorganic phosphate is that an estimation of the plasma phosphate is often required in the study of children's diseases; and it is thought to be desirable to combine this estimation with the phosphatase determination, as may easily be done by making the inorganic phosphate estimation identical with the control for the phosphatase. This is done in some of the glycerophosphate procedures, such as those of Jenner and Kay (1932) and Bodansky (1933). But the use of phenol phosphate for phosphatase determination is greatly to be preferred, because of the greater convenience of the procedure, the much shorter time which is necessary to complete the determination, and the small amounts of blood plasma or serum required.

Two procedures are set out below: in one the determination of liberated phosphate is by the molybdate method of Fiske and Subbarow (1925) with aminonaphtholsulphonic acid as reducing agent; in the other, a more micro procedure, a molybdate-stannous chloride method is used. Both procedures employ the same substrate, buffer, temperature, and hydrolysis time as the phenyl phosphate method in which phenol is determined, so that the conditions are identical with those of the King-Armstrong method as modified by King (1951). The results are identical in theory, since one molecule each of phenol and phosphate are liberated for each molecule of phenyl phosphate hydrolysed, and the comparisons of results obtained by the different procedures has, in fact, shown them to be in excellent agreement.

Methods

Substrate.—This is 0.01 M-disodium phenyl phosphate prepared by dissolving 2.18 g. in 1 litre of distilled water which is heated quickly to the boil to destroy any micro-organisms, and preserved in the ice-box, with a few drops of chloroform added as an antiseptic.†

* The procedures described in this paper were demonstrated to the Biochemical Society and an abstract published in the *Biochemical Journal* (1950, **46**, xxiv).

† If the disodium phenylphosphate contains appreciable inorganic phosphate the solid material should be extracted with 95% alcohol in a Soxhlet.

Buffer (*p*H 10).—This buffer is 0.1 M-sodium carbonate-bicarbonate ($6Na_2CO_3$: $4NaHCO_3$) prepared by dissolving 6.36 g. Na_2CO_3 (anhydrous) and 3.36 g. $NaHCO_3$ in distilled water and diluting to 1 litre.

Buffer (*p*H 4.9).—The second buffer is 0.2 M-sodium acetate-acetic acid (6.5 NaOAc : 3.5 HOAc) prepared by adding 65 ml. 0.2 M-sodium acetate (27.2 g. $C_2H_3O_2Na.3H_2O$ per litre) to 35 ml. 0.2 M-acetic acid (11.3 ml. pure glacial acetic acid per litre).

Trichloroacetic Acid.—7% and 20%.

Molybdate.—Ammonium molybdate, 5 g., dissolved in 100 ml. of approximately 5 N-H_2SO_4 prepared by adding 14 ml. concentrated sulphuric acid, slowly and with stirring, to 86 ml. of distilled water.

Aminonaphtholsulphonic Acid Reducing Agent.—1 : 2 : 4-Aminonaphtholsulphonic acid, 0.2 g., sodium metabisulphite, 12 g., and crystalline sodium sulphite ($Na_2SO_3.7H_2O$), 2.4 g., are dissolved in 100 ml. of distilled water.

Stannous Chloride Reducing Agent.—This is a stock solution prepared monthly and kept in the ice-chest. Stannous chloride ($SnCl_2$) 10 g. is dissolved in 100 ml. of approximately 5 N-HCl (1 : 1 dilution of concentrated reagent hydrochloric acid with distilled water). The reducing agent is a 1 in 20 dilution of the stock solution with 5 N-H_2SO_4, prepared fresh daily.

Stock Standard Phosphate.—Potassium dihydrogen phosphate (pure, A.R.), 2.194 g., is dissolved in distilled water in a 500-ml. flask, made up to the mark, and mixed. A few drops of chloroform should be added to prevent growth of micro-organisms. This solution contains 1.0 mg. phosphorus per ml.

Standard Phosphate Solution (0.004 mg. P in 1 ml.).—A quantity, 2.0 ml., of the stock standard phosphate is accurately measured into a 500-ml. volumetric flask, diluted to the mark with distilled water, well mixed, and preserved with a few drops of chloroform.

Alkaline Phosphatase: Method I*

Test.—Buffer and substrate, 3 ml. of each, are pipetted into a test-tube and warmed in a thermostat water bath at 37° C. for three or four minutes. Plasma or serum, 0.3 ml., is added, the tube stoppered, gently shaken, and left in the bath for exactly 15 minutes. The tube is removed, and 1.2 ml. of 20% trichloroacetic acid quickly added to stop the hydrolysis. The mixture is shaken and filtered or centrifuged clear of precipitated proteins.

Control.—Plasma, 0.3 ml., is added to 6 ml. of water, mixed with 1.2 ml. of 20% trichloroacetic acid, and filtered.

Blank.—Buffer, 3 ml., and 3 ml. of substrate are mixed with 0.3 ml. of water and 1.2 ml. of 20% trichloroacetic acid.

Phosphate Determinations.—To 5 ml. each of the test, control and blank, and of the standard phosphate solution ($\equiv$ 0.02 mg. P), are added 0.8 ml. of the molybdate and 0.2 ml. of the aminonaphtholsulphonic acid reducing agent. The tubes are mixed, and ten minutes allowed for the blue colours to develop, and the reading (extinction) of each is then determined in a photo-electric colorimeter fitted with a red light filter; alternatively the test, control, and blank are read against the standard in a Duboscq colorimeter (preferably with a red light filter over the eye-piece).

Calculation.—Equimolecular amounts of phenol and phosphate are liberated when phenyl phosphate is hydrolysed. For each molecule of phenol of molecular weight 94 there is a molecule of phosphate containing one atom of phosphorus (P) of atomic weight 31. Therefore, there are three times (approximately) as much phenol, by weight, liberated in a

* Determination of phosphate with aminonaphtholsulphonic acid.

given time as there is phosphate expressed as P. The King-Armstrong phosphatase unit is defined as that amount of enzyme which liberates 1 mg. of phenol from phenyl phosphate. It can equally well be defined as that which liberates $\frac{1}{3}$ mg. P. And it is consequently necessary to multiply the amount of P (mg.), liberated in the 15 minutes, by three to obtain a figure equal to the King-Armstrong unit. The units of phosphatase, in any determination, then, are equal to the mg. of phenol, or to three times the mg. of P liberated by the hydrolysis of phenyl phosphate. This is equal to the phenol or phosphate in the test (which consists of that liberated by hydrolysis, *plus* that already present in the plasma (control) and the small amount of free phenol or phosphate in the buffer-substrate blank), *minus* the sum of the phenol or phosphate in the control and the blank. The 5 ml. of filtrate from test and control are equal to 0.2 ml. of plasma. To convert the units of phosphatase to 100 ml. of plasma (basis) it is necessary to multiply by 100 over 0.2.

Phosphatase units per 100 ml. of plasma (or serum)

$$= \frac{\text{Reading of Test} - (\text{Control} + \text{Blank})}{\text{Reading of Standard}} \times 0.02 \times 3 \times \frac{100}{0.2}$$

$$= \frac{T-(C+B)}{S} \times 30 \text{ (when read in a photo-electric colorimeter) ;}$$

$$\text{or, } = \frac{S}{T} - \frac{(S}{(C} + \frac{S)}{B)} \times 30 \text{ (when read in a Duboscq colorimeter).}$$

The inorganic phosphate of the plasma or serum is that contained in the control.

$$\frac{\text{Inorganic P}}{(\text{mg./100 ml.})} = \frac{\text{Reading of Control}}{\text{Reading of Standard}} \times 0.02 \times \frac{100}{0.2}$$

$$= \frac{C}{S} \times 10 \text{ (when read in a photo-electric colorimeter) ;}$$

$$\text{or } \frac{S}{C} \times 10 \text{ (when read in a Duboscq colorimeter).}$$

Alkaline Phosphatase: Micro Method II*

Test.—Quantities, 1 ml. each of buffer and substrate, are pipetted into a test-tube and warmed in a water bath at 37° C. for 3 or 4 minutes ; 0.1 ml. of plasma or serum is added, stoppered, mixed, and kept at 37° for 15 minutes. Seven per cent trichloroacetic acid, 2.9 ml., is added, the tube removed and shaken, and the mixture filtered or centrifuged.

Control.—The control is prepared by treating 2 ml. water and 0.1 ml. of plasma with 2.9 ml. of 7% trichloroacetic acid, mixing, and filtering.

Blank.—The blank consists of buffer and substrate, 1 ml. each, 0.1 ml. water, and 2.9 ml. of 7% trichloroacetic acid.

Standard.—The standard consists of the standard phosphate solution (1 ml. $\equiv$ 0.004 mg. P), *plus* 1 ml. of 7% trichloroacetic acid.

Phosphate Determinations.—The standard, and 2 ml. each of the test, control, and blank are diluted with 3 ml. of water, and treated with 0.8 ml. of molybdate. They are well mixed and 0.2 ml. of stannous chloride added, and mixed again. The colours are read after 15 minutes. With a photo-electric colorimeter the zero is set with a reagent blank consisting of 4 ml. water, 1 ml. of 7% trichloroacetic acid, 0.8 ml. molybdate, and 0.2 ml. stannous chloride.

Calculation.—The 2 ml. of test and control represent 0.04 ml. of plasma, and, therefore, phosphatase units per 100 ml.

*Determination of phosphate with stannous chloride.

$$= \frac{\text{Reading of test} - (\text{Control} + \text{Blank})}{\text{Reading of Standard}} \times 0.004 \times 3 \times \frac{100}{0.04}$$

$$= \frac{T-(C+B)}{S} \times 30 \text{ (when read in a photo-electric colorimeter);}$$

$$\text{or, } = \frac{S}{T} - \frac{(S}{(C} + \frac{S)}{B)} \times 30 \text{ (when read in a Duboscq colorimeter)}$$

$$\text{Inorganic P (mg./100 ml.)} = \left[\frac{\text{Reading of Control}}{\text{Reading of Standard}} \times 0.004 \times \frac{100}{0.04}\right.$$

$$= \frac{C}{S} \times 10 \text{ (when read in a photo-electric colorimeter)}$$

$$\text{or, } \frac{S}{C} \times 10 \text{ (when read in a Duboscq colorimeter)}$$

The Stannous Chloride Phosphate Method

Stannous chloride is not as good a reducing agent for the colorimetric determination of phosphate as aminonaphtholsulphonic acid, but it yields much more blue colour from the same amount of phosphate. It is therefore preferred for minimal micro-methods. The colours

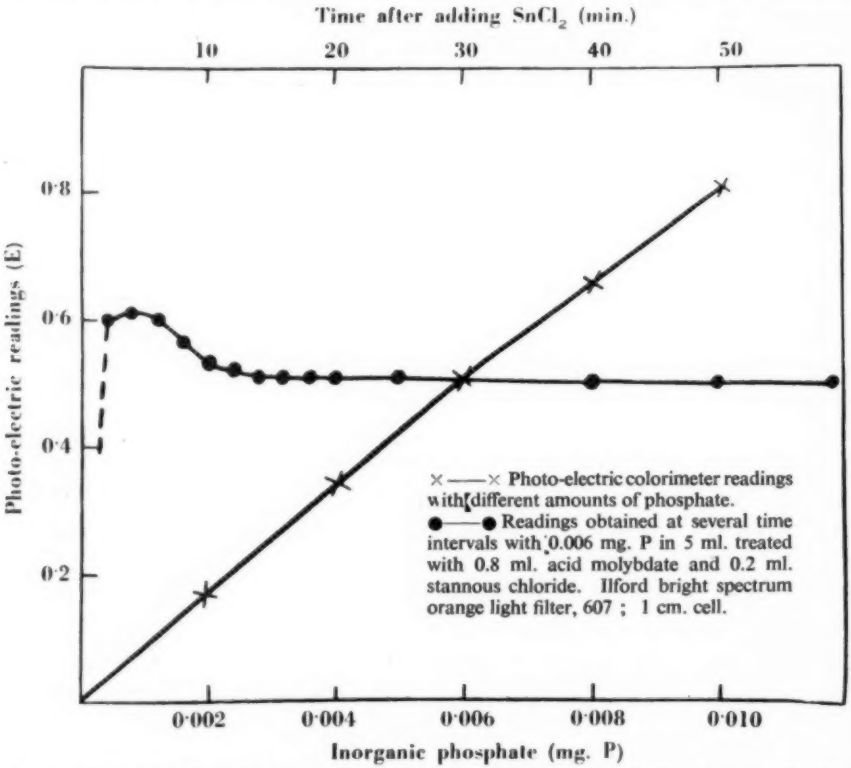

FIG. 1.—Proportionality of colour and rate of colour development with the stannous chloride-molybdate method for inorganic phosphate.

produced with it may not always obey Beer's law (Bodansky, 1937). It is more sensitive to changes in the concentration of acid present than the photographic reducing agents, and there may be non-specific reduction of the molybdate if the mixtures are not well mixed before and immediately after adding the $SnCl_2$ solution. But if the conditions of the test and the concentrations of reagents are carefully chosen, it is possible to obtain reproducible and proportional results (Fig. 1). The accuracy of the procedure is sufficient for most purposes.

Results

Alkaline Phosphatase.—Comparisons between these two phosphate procedures, on the one hand, and the phenol method of determining phosphatase on the other, have yielded results which agree very well. These are set out in Tables I and II. It is apparent that with phenyl phosphate as substrate the same result, or nearly the same result, is obtained by all three procedures, and that it makes no difference whether phenol or phosphate is determined and taken as the measure of the enzyme activity. The errors are not greater than one might expect in comparing any two

TABLE I

DETERMINATION OF SERUM ALKALINE PHOSPHATASE BY PHENOL AND PHOSPHATE METHODS

Case	King-Armstrong Phenol Method (units/100 ml. serum)	Phosphate Method (Aminonaphtholsulphonic Acid)	
		Phosphatase (units/100 ml. serum)	Inorganic P (mg./100 ml.)
Normal adult	7.0	6.3	2.9
,, ,,	9.5	8.8	4.1
,, child	9.8	10.0	3.6
,, ,,	9.8	10.6	4.2
,, ,,	13.0	12.7	4.3
Rickets	11.0	11.2	4.5
,,	36.5	32.5	3.5
,,	38.0	35.7	2.5
,,	43.5	40.5	2.0
,,	45.5	47.5	1.6
,,	57.5	60.0	1.8
,,	65.5	65.0	1.6
,,	67.5	63.5	1.8
,,	116.5	120.0	1.7
? Hepatitis	7.0	6.0	3.2
,,	14.0	13.2	3.4
? ,,	14.4	15.0	5.2
,,	16.5	14.8	3.0
,,	19.0	17.5	3.5
,,	19.0	18.8	3.0
,,	20.5	21.4	3.6
,,	27.5	25.8	—
,,	28.0	27.5	4.0
Cirrhosis	6.4	6.0	2.8
,,	12.0	11.5	3.8
Obstructive jaundice	34.5	32.3	—
,, ,,	46.5	47.1	4.1
Splenomegaly ,,	30.0	30.5	3.1
Paget's disease	56.0	53.2	2.9
Prostatic carcinoma	22.1	20.8	4.1

TABLE II

DETERMINATION OF SERUM ALKALINE PHOSPHATASE BY PHENOL AND PHOSPHATE METHOD

Case	King-Armstrong Phenol Method (units/100 ml. serum)	Micro Phosphate Method using Stannous Chloride	
		Phosphatase (units/100 ml. serum)	Inorganic P (mg./100 ml.)
Normal adult	6.4	6.2	4.0
,, ,,	8.2	6.8	—
,, ,,	8.8	9.3	3.2
,, ,,	10.0	11.4	2.9
,, ,,	10.2	9.0	3.3
,, ,,	15.2	14.8	3.0
Normal child	11.2	12.0	5.6
,, ,,	12.8	12.4	5.8
Rickets	26.6	27.5	4.0
,,	40.0	37.5	2.4
,,	71.0	66.5	1.3

different colorimetric procedures. And when it is remembered that errors of measurement, dilution, and precipitation of protein by two different means are included with those due to the production of colours by two different chemical reactions, then it is seen that the agreement is all that can be desired.

The micro procedure (Method II) using only 0.1 ml. of serum, and stannous chloride as the reducing agent, is a useful modification where only small amounts of blood are available, as may frequently be the case in the investigation of infants. And there is no reason why the method should not be made still more minimal, with, say, 0.05 ml. of serum or plasma, by employing small test-tubes and colorimeter cells and cutting all quantities by half.

Inorganic phosphate results (expressed as mg. P/100 ml. of serum) for the same blood sera are included in the tables; these are the figures obtained from the controls in the phosphatase methods.

Acid Phosphatase.—It is perhaps worth pointing out that either of these modifications of the phosphatase determination can be applied to the estimation of acid phosphatase (Gutman and Gutman, 1940; King, 1951) just as well as to alkaline

TABLE III

DETERMINATION OF SERUM ACID PHOSPHATASE BY PHENOL AND PHOSPHATE METHODS (AMINONAPHTHOLSULPHONIC ACID PROCEDURE)

Case	Method (units/100 ml. serum)	
	Phenol	Phosphate
Normal	0.9	1.5
,,	1.4	2.6
,,	3.9	4.5
Prostatic carcinoma	8.3	7.1
,, ,,	14.0	13.9
,, ,,	15.0	15.9
,, ,,	32.8	34.2

phosphatase, if it is desired to carry out the estimations by means of phosphate determination instead of the usually used phenol determination. But it is necessary to employ an acetic acid buffer (*p*H 4.9) instead of the usual citric acid buffer, since the latter interferes with the molybdate procedures for determining inorganic phosphate. The mixtures of substrate, buffer (acetic), and serum are as above, the time of 38° incubation is one hour, and the phosphate determinations as described. With the aminonaphtholsulphonic method we have obtained the type of agreement between it and the phenol acid phosphatase procedure (Gutman and Gutman, 1940; King, 1951) which is illustrated in Table III.

Summary

Two modifications of the King-Armstrong phenyl phosphatase procedure for serum alkaline phosphatase determinations have been described.

Since equimolecular quantities of phenol and inorganic phosphate are liberated in the hydrolysis of phenyl phosphate, it has been possible to substitute determinations of phosphate for those of phenol, which have hitherto been used.

In the first method 0.3 ml. of plasma or serum is employed, and the inorganic phosphate method of Fiske and Subbarow (1925) using aminonaphtholsulphonic acid as the reducing agent.

In the second method, which is a micro modification, 0.1 ml. of plasma or serum is used, and the stannous chloride-molybdate method of phosphate determination.

Results obtained by these " phosphate " procedures are in good agreement with those by the " phenol " method of determining phosphatase activity.

Determinations of the inorganic phosphate of the plasma or serum are included as part of the phosphatase determination.

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MICRO-ESTIMATION OF PLASMA IRON WITH ORTHOTOLIDINE

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The estimation of plasma or serum iron and of the iron-binding capacity of the plasma is becoming an increasingly important diagnostic procedure for the clinical haematologist (Rath and Finch, 1949; Cartwright and Wintrobe, 1949). Current methods for this estimation are time-consuming and difficult to carry out, and their use has therefore been confined mainly to the research laboratory. The test to be described is so simple that it can be used as a routine in any laboratory. It overcomes in a reliable way the difficulty of rendering the glassware iron-free, and it requires less than 1 ml. of plasma for a double estimation, an important consideration in work on children and small animals.

o-Tolidine (4, 4′-diamino-3, 3′-dimethyl-diphenyl) was first used for the demonstration of blood in the urine by Ruttan and Hardisty in 1912. The procedure has lately been thoroughly standardized by Zwarenstein (1949). In carrying out the test for blood in the urine it was soon found that the slightest contamination with iron produced false positive results. From this observation the method for the plasma iron estimation was developed.

Reagents

The following reagents are required.

1. Glass-distilled Water.

2. Standard Iron Solution.—Wong's solution is convenient.

Crystallized ferrous ammonium sulphate (dried to constant weight), 0.7 g., is dissolved in about 50 ml. of glass-distilled water. To it is added 20 ml. of 10 % iron-free sulphuric acid, the solution is warmed slightly, and then is added decinormal solution of potassium permanganate to oxidize the ferrous salt completely. It is diluted to 1,000 ml. with water. One ml. contains 100 μg. of iron.

The working solution is prepared by diluting 1 ml. of the standard solution to 100 ml. with water, so that 1 ml. contains 1 μg. of iron.

3. Trichloracetic Acid Solution, 50% v/v.—Trichloracetic acid is mixed with excess concentrated sulphuric acid and distilled in an all-glass distillation apparatus into a known volume of water in a measuring cylinder. After the distillation the volume is adjusted with water to make a 50% v/v solution.

4. o-Tolidine Solution (0.05% w/v).—Pure (nearly white) *o*-tolidine hydrochloride, 0.5 g., is dissolved in 1,000 ml. of water. The solution becomes slightly brownish on standing, but retains its effectiveness for months, especially if stored in the dark in a refrigerator.

During this investigation the laboratory ran out of pure *o*-tolidine hydrochloride and the sulphate had to be substituted temporarily. This substance is less soluble, and in a 0.05% w/v solution a precipitate remains. However, if the solution is kept in a tall narrow container the precipitate settles firmly on the bottom and the supernatant fluid is as effective as the hydrochloride solution.

5. Acid-peroxide Solution.—Equal volumes of glass-distilled glacial acetic acid and 3% hydrogen peroxide, freshly diluted from 30%, are mixed. This stock solution lasts for many months. The working solution is prepared by diluting 2 ml. of stock solution to 10 ml. with water. It was found that different batches of alleged 30% hydrogen peroxide differed markedly in their true strength. It may therefore be necessary to adjust the working solution in such a way that in carrying out the test the blank remains nearly colourless and the colour with known amounts of iron falls inside the best working range of the colorimeter used.

Cleaning of Glassware

All glassware used in the test is cleaned in ordinary bichromate cleaning fluid and rinsed in tap water and glass-distilled water. Any trace of iron left is now removed by filling the glassware with *o*-tolidine solution, adding a few drops of trichloracetic acid and a few drops of acid-peroxide, and keeping the glassware in a boiling water bath for about 10 minutes. The glassware is then drained and is ready for use. The ordinary test-tubes employed in the test can be used again after each estimation simply by draining and without further cleaning. Before this procedure was adopted it was found impossible to obtain reproducible results. With this cleaning method the results have been consistently accurate with known amounts of iron.

For each solution a separate pipette is kept in a holster made of a large piece of filter paper rolled into a tube.

Procedure

Contamination of the blood is avoided by collecting it without the use of a syringe. An intravenous steel needle is inserted into a convenient vein and about 0.5 ml. of blood is allowed to run away. About 3 ml. of blood is run into an *o*-tolidine-cleaned Kahn tube containing a drop of heparin solution. The solution is mixed, and centrifuged at 3,000 r.p.m. for 30 minutes.

Plasma, 0.25 ml., is transferred into one Kahn tube and 0.50 ml. into another, and 1.75 ml. of water to the first tube and 1.50 ml. to the other is added.

To both tubes is added 0.2 ml. of trichloracetic acid solution; the tubes are shaken carefully and placed in a boiling water bath for about three minutes.

The tubes are centrifuged at 3,000 r.p.m. for about five minutes.

These quantities are added to five ordinary test-tubes in sequence: 1 ml. water; 0.75 ml. water + 0.25 ml. iron solution; 0.50 ml. water + 0.50 ml. iron solution; 1.1 ml. supernatant fluid from the first Kahn tube; 1.1 ml. supernatant fluid from the second Kahn tube. To the first three tubes is added 0.1 ml. trichloracetic acid solution. To all tubes is added 5 ml. *o*-tolidine solution. To all tubes is added 0.1 ml. acid-peroxide solution.

The tubes are placed in a boiling water bath for 10 minutes. A golden-brown colour develops, the depth depending on the amount of iron present.

The tubes are cooled to room temperature and the depth of colour estimated in a photoelectric colorimeter using the first tube as the blank.

In this work a Klett-Summerson colorimeter with the blue filter No. 42 (spectral range 400–465 mμ) was used. Calculation:

$$\text{Calculation: } \frac{\frac{(P_1 \times 800 + P_2 \times 400)}{2}}{\frac{(I_1 \times 4 + I_2 \times 2)}{2}} = \frac{4P_1 + 2P_2}{2I_1 + I_2} \times 100 = \mu\text{g./100 ml.}$$

where P_1 = reading of test-tube No. 4 (0.125 ml. plasma)
P_2 = reading of test-tube No. 5 (0.25 ml. plasma)
I_1 = reading of test-tube No. 2 (0.25 μg. iron)
I_2 = reading of test-tube No. 3 (0.50 μg. iron)

The purpose of using different amounts of both iron solution and plasma in the duplicate estimations included in one test is, first, to safeguard against contamination, which is a constant danger in all plasma iron estimations; secondly, to assure that sufficient *o*-tolidine is present to develop the full colour with larger amounts of iron; and, thirdly, to safeguard against incomplete precipitation of the plasma proteins or incomplete recovery of the protein-bound iron.

The number of tubes included in the test does not add to the labour involved, and any number of plasma iron estimations can be performed at one time, depending on the size of the water baths used.

Total Iron-binding Capacity of the Plasma

This is determined by the method suggested by Cartwright and Wintrobe (1949). After the collection of the blood as in the first step of the procedure described a supersaturating amount of iron is injected through the needle, and five minutes later another 3 ml. of blood collected from a different vein. The plasma iron of this blood is estimated with the first sample and is equal to the total iron-binding capacity of the patient's plasma.

In this investigation 7 ml. of ferrocalcium, "sandoz" (10 mg. of iron), were used in adult patients, and correspondingly smaller amounts in children.

Results

Correlation between Iron Content and Colour Development.—Fig. 1 shows that in the range from 0 to 1.0 μg. of iron the correlation between iron content and colorimeter reading is a straight line. With the quantities of plasma used in the test it is thus possible to estimate from 0 to 800 μg. of iron per 100 ml. of plasma, a range that covers all possible pathological values.

Specificity of the Reaction.—To a number of test-tubes were added 0.5 ml. of iron solution and 0.5 ml. of different solutions containing the following substances: Al, Ba, Bi, Br, Ca, Cd, Co, Cu, Hg, K, Li, Mg, Mn, Na, Ni, Pb, Sb, Sn, Sr, Zn, urea, uric acid, and creatine.

In those cases in which the concentration of the particular substance in human blood was known, the solution was adjusted to a value corresponding to about 10 times the value likely to be found in the blood. In no case did the readings of the colorimeter differ more than five divisions, which was within the error of the method. Iodine and bromine are known to produce a colour with *o*-tolidine, but the amounts necessary to interfere with the plasma iron estimation are far in excess of those found in the blood.

Oxalate, even in small amounts, completely inhibits the colour development. Oxalated blood cannot be used in this test, and if heparin is not available the iron

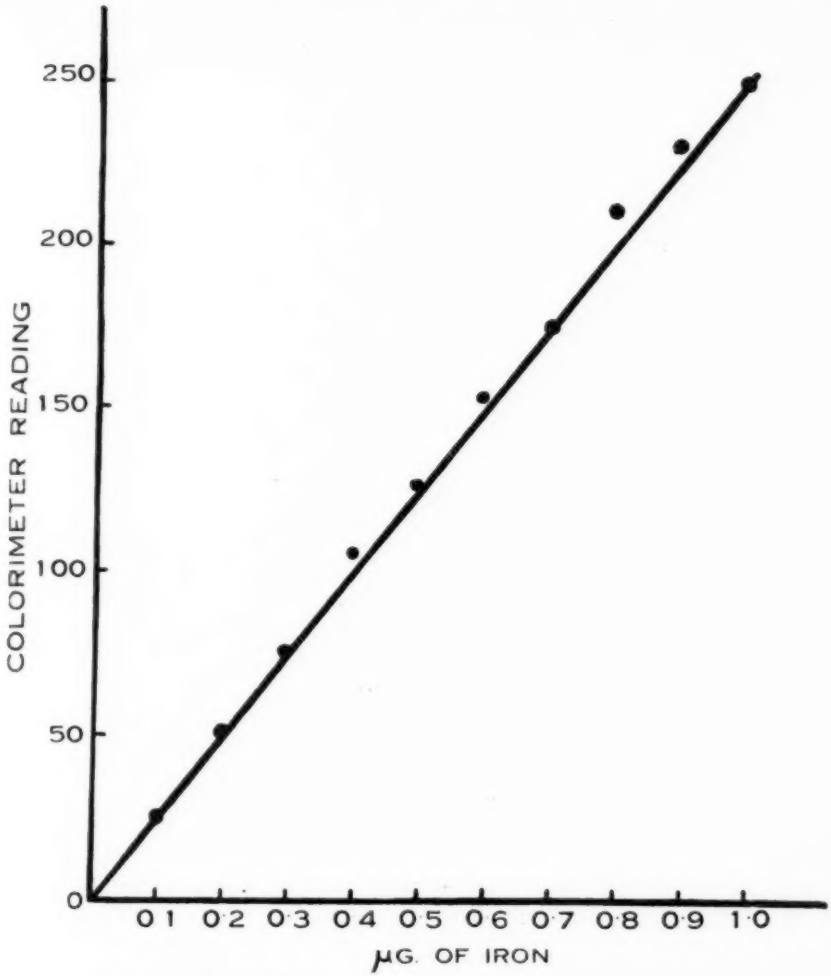

FIG. 1.—Correlation between iron content and colorimeter reading.

estimation must be done on serum which gives the same results as plasma. Serum is not used as a routine, as the loosening of the clot from the tube before centrifuging may introduce contamination.

Ascorbic acid is known to inhibit the *o*-tolidine test for blood in the urine (Barach and Pennock, 1940). In the case of plasma iron the amounts of ascorbic acid necessary to inhibit the colour development appreciably are of the order of 10 mg. per 100 ml. of plasma, values that again lie well above those found in the blood.

Recovery of the Plasma Iron.—Kitzes, Elvehjem, and Schuette (1944), who also precipitated the proteins by heat and trichloracetic acid, found that a single washing gave full recovery of the liberated iron.

During the development of the present method washing of the precipitate with *o*-tolidine solution was first resorted to, but it was soon found that, due to the rise in the *p*H during the washing, some of the protein went into solution again and interfered with the colour development. Washing with trichloracetic acid solution was then tried, but two or even three washings did not increase the amount of iron recovered and only added to the manipulative difficulties and the danger of contamination.

Experiments aiming at the recovery of iron added to the plasma have also shown that the recovery is complete. Two examples are given in Table I.

TABLE I

RECOVERY OF IRON ADDED TO PLASMA

	Tube No.	Test Solution	Colorimeter Reading	Total Iron Found (μg.)	Calculated Value (μg.)
(a)	1	1 ml. water	0	(0)	
	2	0.75 ml. water + 0.25 ml. iron solution	56	(0.25)	
	3	0.50 ml. water + 0.50 ml. iron solution	110	(0.50)	
	4	0.875 ml. water + 0.125 ml. plasma extract	36	0.16	
	5	0.75 ml. water + 0.25 ml. plasma extract	73	0.33	
	6	0.75 ml. water + 0.10 ml. plasma extract + 0.15 ml. iron solution	68	0.31	0.28
	7	0.75 ml. water + 0.15 ml. plasma extract + 0.10 ml. iron solution	67	0.30	0.30
	8	0.75 ml. water + 0.20 ml. plasma extract + 0.05 ml. iron solution	70	0.32	0.31
(b)	1	1 ml. water	0	(0)	
	2	0.75 ml. water + 0.25 ml. iron solution	75	(0.25)	
	3	0.50 ml. water + 0.50 ml. iron solution	150	(0.50)	
	4	0.875 ml. water + 0.125 ml. plasma extract	63	0.21	
	5	0.75 ml. water + 0.25 ml. plasma extract	130	0.43	
	6	0.625 ml. water + 0.125 ml. plasma extract + 0.25 ml. iron solution	145	0.48	0.47
	7	0.75 ml. water + 0.125 ml. plasma extract + 0.125 ml. iron solution	100	0.333	0.345
	8	0.875 ml. water + 0.075 ml. plasma extract + 0.05 ml. iron solution	52	0.17	0.17

The Stability of the Colour.—To determine the stability of the colour the test tubes from one determination were kept at room temperature for 24 hours. The results are given in Table II.

TABLE II

THE STABILITY OF THE COLOUR

Tube No.	Colorimeter Reading 1 Hour after Removal from Water Bath	Colorimeter Reading 24 Hours Later
1	0	0
2	65	65
3	135	140
4	50	50
5	99	95

Occasionally a fine brown precipitate separates out on prolonged standing. Placing the tubes in the water bath re-dissolves this precipitate, and, on cooling, the original colorimeter readings are again obtained.

TABLE III

THE ACCURACY OF THE METHOD

Tube No.	Test Solution	Colorimeter Reading	Plasma Iron (μg. per 100 ml.)
1	Water	0	
	Iron solution equivalent to:		
2	0.25 μg. iron	80	
2a	0.25 ,, ,,	80	
3	0.5 ,, ,,	160	
3a	0.5 ,, ,,	162	
	Supernatant fluid equivalent to:		
4	0.125 ml. plasma	54	135
5	0.125 ,, ,,	53	133
6	0.125 ,, ,,	53	133
7	0.125 ,, ,,	54	135
8	0.125 ,, ,,	52	130
9	0.125 ,, ,,	51	129
10	0.125 ,, ,,	55	137
11	0.125 ,, ,,	57	142
12	0.125 ,, ,,	55	137
13	0.125 ,, ,,	53	133
14	0.25 ,, ,,	115	143
15	0.25 ,, ,,	110	137
16	0.25 ,, ,,	105	131
17	0.25 ,, ,,	109	136
18	0.25 ,, ,,	111	139
19	0.25 ,, ,,	107	134
20	0.25 ,, ,,	111	139
21	0.25 ,, ,,	113	141
22	0.25 ,, ,,	110	137
23	0.25 ,, ,,	115	143

Mean	136 μg./100 ml.
Standard deviation	4 μg.
Coefficient of variation	3%
Significant difference ($\pm 2\sqrt{2}s^2$)	±12 μg.
Range ($\pm$ 2s)	128–144 μg.

The Accuracy of the Method.—Twenty estimations were carried out simultaneously on the same plasma, ten on 0.25 ml. and ten on 0.5 ml. of plasma. The results are tabulated in Table III.

The taking of the blood from the patient, its transport, and centrifugation must add to the error of the method, as it must to all other methods, but the coefficient of variation is so small, expecially in view of the large normal range of plasma iron from 100 to 200 μg. per 100 ml., that the accuracy remains highly satisfactory.

Plasma Iron Values with the o-Tolidine Method.—In 25 normal adults the plasma iron varied between 90 and 210 μg. per 100 ml. with a mean of 140 μg. The total iron-binding capacity fell between 300 and 400 μg. per 100 ml. with an average of 330 μg. The high plasma iron in pernicious anaemia and the low values in iron-deficiency anaemia of infection have been similar to those reported in the literature.

Direct comparison with other methods was attempted. σ, α^1-Dipyridyl was not available. The potassium thiocyanate method of Tötterman (1949) was too insensitive in our hands or with our colorimeter to give readable results. The method of Vannotti and Delachaux (1949) gave the following values for their fraction A + B on the same plasma, using 4 ml. of plasma for each test: 185 μg. and 162 μg. per 100 ml., whereas the *o*-tolidine method on this plasma gave the values of 171 μg. and 175 μg. per 100 ml.

Great difficulty was experienced in the precipitation of the proteins with the method of Vannotti and Delachaux. The impression remained that at least the first three of their plasma iron fractions were related to such precipitation difficulties, and the later work of Surgenor, Koechlin, and Strong (1949), Rath and Finch (1949), and Cartwright and Wintrobe (1949) would also indicate that such fractions are artifacts and of little pathological importance.

There seems little doubt that the *o*-tolidine method estimates the iron bound to the β_1-globulin of the plasma and therefore the iron of pathological significance.

Summary

A method is described for the estimation of plasma iron, including the total iron-binding capacity of the plasma, which is simple, rapid, and reliable.

The method is more sensitive than any previously described, requiring less than 1 ml. of plasma for a duplicate estimation.

The plasma iron values found by this test correspond to those obtained with other methods.

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TECHNICAL METHODS

A PHOTOMETRIC METHOD FOR THE ESTIMATION OF SODIUM AND ITS APPLICATION TO SERUM SODIUM DETERMINATION

BY

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Most methods for the colorimetric estimation of sodium depend upon the precipitation of the metal as sodium zinc uranyl acetate, a reaction first proposed by Barber and Kolthoff (1928). The precipitated salt, after washing, is treated in various ways to give coloured solutions. There are thus three distinct stages which have to be considered in the estimation: (1) precipitation, (2) washing of the precipitate, and (3) final colour.

Precipitation of Sodium Zinc Uranyl Acetate

Among the precipitating reagents used are a 50% alcoholic solution (McCance and Shipp, 1931), and aqueous solutions (Barber and Kolthoff, 1928; Butler and Tuthill, 1931; Weinbach, 1935) of zinc uranyl acetate, all in the presence of acetic acid. The triple salt is precipitated in a number of ways. McCance and Shipp (1931) treated 1 part of aqueous sodium solution with 10 parts of alcoholic zinc uranyl acetate at 0° C. for one hour, while Albanese and Lein (1948) used 1 to 4 parts of aqueous sodium solution and 10 parts of alcoholic reagent at 4° C. for 1 hour. Of the aqueous reagents, that of Weinbach has been used most frequently. In his original method Weinbach treated 1 part of aqueous solution and 5 parts of aqueous reagent with 3.5 parts of absolute alcohol over a period of half an hour. Bradbury (1946) used 1 part of aqueous sodium solution, 4 parts of aqueous reagent (Weinbach), and 2 parts of 95% alcohol, and allowed the mixture to stand at room temperature for 20 to 30 minutes. Fowweather and Anderson (1948) found the method of Bradbury unsatisfactory and used 1 part of aqueous solution, 2 parts of aqueous reagent, 3 parts of absolute alcohol, and 2 parts of water at 3° C. for 2 hours. In the present method a mixture of 1 part of aqueous sodium solution, 2 parts of absolute alcohol, and 3 parts of alcoholic zinc uranyl acetate (prepared according to McCance and Shipp) has been used for the precipitation at room temperature over a period of two hours.

Washing the Precipitate

In many methods a saturated alcoholic solution of sodium zinc uranyl acetate is used as washing liquor, although saturated solutions of the triple salt in acetone and

glacial acetic acid have also been used. In 1938, however, Hoffman and Osgood showed that saturated solutions of the triple salt in alcohol, acetone, or glacial acetic acid produced precipitates of uranium salts when brought into contact with zinc uranyl acetate. Since the intensity of the colour reactions of sodium zinc uranyl acetate is dependent on the amount of uranium present, high results will be obtained if any of these washing liquors is used with the alcoholic and aqueous reagents previously mentioned. This difficulty was, apparently, overcome by Noyons (1939), using an aqueous solution of zinc uranyl acetate but containing a greater proportion of acetic acid than the other aqueous reagents. The triple salt, however, is not precipitated completely under 24 hours. A further disadvantage of this method is that the precipitate, consisting of larger crystals than those obtained using alcoholic zinc uranyl acetate, is more difficult to pack on centrifuging, with a consequent greater possibility of mechanical loss when the supernatant solution is removed. Darnell and Walker (1940) used ethyl acetate-acetic acid mixture for washing. This is somewhat unpleasant, and, in addition, two extra washings with ether are necessary in order to remove the acetic acid, thus greatly lengthening the procedure. A saturated alcoholic solution of magnesium acetate has been found satisfactory as a wash liquor in the method to be described. This solution (5 ml.) on adding to alcoholic zinc uranyl acetate solution (0.5 ml.) gives no precipitate, and on shaking with sodium zinc uranyl acetate and filtering gives no colour with sodium salicylate.

Final Colour

McCance and Shipp (1931) dissolved the precipitate of sodium zinc uranyl acetate in water, added acetic acid and potassium ferrocyanide, and obtained a plum-red solution. Owing to the ease with which this solution becomes cloudy, fallacious results may be obtained using photometric technique for colour measurement. Hoffman and Osgood added ammonium thiocyanate to the dissolved triple salt, but the colour does not obey Beer's law and is not very sensitive.

Fredericq (1942) treated a solution of the triple salt with alizarin and obtained a violet solution; he reported an error of 1–8% for 400–25 μg. Na.

In 1946 Bradbury measured the residual colour of the zinc uranyl acetate after the precipitation of the triple salt, and in 1948 Albanese and Lein dissolved the triple salt in water and measured the intensity of the resulting yellow solution. Both methods, however, lack sensitivity and are liable to large experimental errors when used on the microscale in routine work. In the method of Darnell and Walker the precipitate of sodium zinc uranyl acetate is dissolved in water and treated with sulphosalicylic acid and sodium acetate. The deep yellow colour obtained, although stable, does not follow exactly Beer's law, and a calibration curve must be used.

The method developed in this laboratory produces an orange colour. The triple salt, dissolved in water, is treated with 1% sodium salicylate. This solution must be prepared accurately, since the colour varies slightly with the concentration of salicylate. The colour obtained is stable for at least 18 hours and is linear over a range 57.5–287.5 μg. Na. If a trichloracetic acid filtrate equivalent to 0.05 ml. serum is used, this range corresponds to 115–575 mg. Na/100 ml. The curve obtained, although linear, cannot be extended through the origin, so that colorimetric readings are not exactly proportional to the concentration of sodium present. The use of a

calibration curve can easily be avoided by reading the colours against an arbitrary blank instead of water, when exact proportionality of colorimetric readings and concentration is obtained. Uranyl acetate (0.2%) was chosen as an arbitrary blank, but any stable yellow solution of suitable strength can be used.

Experimental

Reagents.—The following reagents were used.

Zinc Uranyl Acetate.—Uranyl acetate, 50 g., $UO_2(CH_3COO)_2 2H_2O$, is dissolved in 250 ml. boiling water containing 10 ml. of glacial acetic acid. In another flask 150 g. zinc acetate, $Zn(CH_3COO)_2 2H_2O$, is dissolved in 250 ml. boiling water containing 5 ml. glacial acetic acid. The boiling solutions are mixed and again heated just to boiling. After standing overnight at room temperature, the solution is filtered and mixed with an equal volume of absolute alcohol. Some triple acetate is added to ensure saturation, and the reagent is kept at room temperature. A portion is filtered or centrifuged before use.

Alcohol.—Absolute.

Sodium Zinc Uranyl Acetate, $(UO_2)_3\ Zn.Na(CH_3COO)_9.6H_2O$.—Fifty ml. of the zinc uranyl acetate reagent is added to about 100 ml. of 50% alcohol saturated with sodium chloride. After standing, the precipitate is filtered by suction through a sintered glass filter, washed thoroughly with alcohol, and finally with ether. The last traces of ether are removed in a desiccator. A quantity of 1.537 g. of this triple salt is dissolved in water and the volume made up to 200 ml. This is a stock solution and 1 ml. is equivalent to 0.115 mg. Na.

Standard Sodium Chloride Solution A (containing 2.30 mg. Na/ml.)—Analytical dry sodium chloride, 0.585 g., is dissolved in water, and the volume made up to 100 ml.

Standard Sodium Chloride Solution B (containing 0.75 mg. Na/ml.)—Analytical dry sodium chloride, 0.191 g., is dissolved in water and the volume made up to 100 ml.

Magnesium Acetate.—Saturated alcoholic solution, which is filtered before use.

Sodium Salicylate (1%).—A quantity of 1.0 g. is dissolved in water and the volume made up to 100 ml.

Trichloracetic Acid (7%).—A quantity of 7.0 g. is dissolved in water and the volume made up to 100 ml.

Uranyl Acetate (0.2%).—A quantity of 0.2 g. is dissolved in water and the volume made up to 100 ml.

Method

Principle.—Precipitates of triple acetate from standard sodium chloride solutions, after washing with a saturated alcoholic solution of magnesium acetate, are treated with sodium salicylate. The colours produced are compared with those obtained using standard solutions of triple acetate and sodium salicylate.

Procedure.—Dilutions of the standard NaCl solution A are made and 0.2-ml. volumes, containing 57.5–287.5 μg. Na, are precipitated with the zinc uranyl acetate reagent in the proportions 1 part NaCl solution, 2 parts absolute alcohol, and 3 parts of reagent in pyrex centrifuge tubes. The tubes are allowed to stand at room temperature for two hours with occasional mixing of the contents; they are then centrifuged for 10 minutes. The supernatant solutions are decanted and the tubes allowed to drain on filter paper. Absolute alcohol, 5 ml., saturated with magnesium acetate is added down the sides of the tubes, and the precipitates are very thoroughly mixed with the wash liquor by means of a fine glass rod. After centrifuging for 10 minutes, the supernatant solutions are decanted and the tubes allowed to drain as before. The washed precipitates are dissolved in 5 ml. water and the colour developed by the addition of 5 ml. sodium salicylate. Dilutions of the stock

triple acetate solution (1/10, 1/5, and 1/2) are made in volumetric flasks and volumes of 5 ml. treated with 5 ml. sodium salicylate.

Reading of Colour.—The orange colour is read in a Spekker absorptiometer using Ilford blue filter 602 and 0.5-cm. cells against comparison cells containing 0.2% uranyl acetate. With an " EEL " photo-electric colorimeter the colours can be read directly using filter 622 and micro-tubes (2 ml.).

The results are given in Table I and the absolute linearity of the method is readily seen.

TABLE I

COMPARISON OF RESULTS FROM PRECIPITATED SODIUM CHLORIDE SOLUTIONS AND STANDARD SOLUTIONS OF SODIUM ZINC URANYL ACETATE

Sodium concentration (μg.)		57.5	115.0	172.5	230.0	287.5
Equivalent serum Na. (mg./100 ml.)		115.0	230.0	345.0	460.0	575.0
Spekker readings triple acetate*		0.138	0.278	0.416	0.558	0.689
Spekker readings NaCl Sol. A.†		0.137	0.278	0.415	0.556	0.686
Range of readings	Minimum	0.132	0.275	0.411	0.550	0.679
	Maximum	0.140	0.283	0.419	0.559	0.693
Standard deviation	±	0.0023	0.0027	0.0031	0.0028	0.0050

*Mean of 4 readings (0.2% uranyl acetate in comparison cell). †Mean of 12 estimations (0.2% uranyl acetate in comparison cell).

Serum Sodium Estimation.—Serum or plasma, 0.5 ml., is treated with 1.5 ml. trichloracetic acid, mixed well, and filtered, and 0.2 ml. of the clear filtrate (= 0.05 ml. serum) is precipitated in the same way as the standard sodium chloride solutions. For ease of calculation the colour is compared with that produced by 0.2 ml. of standard NaCl solution B treated in a similar manner.

$$\text{Serum sodium is given by } \frac{\text{Reading of unknown}}{\text{,, ,, standard}} \times 300$$

Recovery Experiments.—The results obtained when known quantities of sodium are added to plasma are given in Table II.

TABLE II

RECOVERY OF ADDED SODIUM FROM PLASMA

Plasma No.	Sodium Added (mg./100 ml.)	Sodium Found (mg./100 ml.)	Sodium Calculated (mg./100 ml.)
1	0	342	—
	10	353	352
	20	360	362
	40	382	382
2	0	330	—
	90	425	420
	90	423	420
	90	416	420
	120	444	450
	120	446	450
3	0	335	—
	100	435	435
	100	441	435
	150	480	485
	200	529	535

Summary

A simple, accurate method for the photometric estimation of sodium, depending on the interaction of sodium zinc uranyl acetate with sodium salicylate, is described. The colour obeys Beer's law, when read against an arbitrary blank, over a range 57.5–287.5 μg. Na, corresponding to a serum sodium range of 115–575 mg./100 ml.

I wish to thank Dr. A. J. McCall for reading the manuscript, and I am indebted to Mrs. C. E. D. Jackson and Mr. G. W. Dalzell, A.I.M.L.T., for technical assistance.

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A SCREENING TEST FOR HETEROPHILE ANTIBODIES IN INFECTIOUS MONONUCLEOSIS

BY

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When Paul and Bunnell (1932) demonstrated the increased titre of heterophile antibodies in the sera of patients with infectious mononucleosis they did so by using the technique devised by Davidsohn (1929, 1930, 1933) when he was investigating these antibodies in serum sickness. It was also used by other workers who sought to confirm the findings of Paul and Bunnell (Bernstein, 1934; Van Ravenswaay, 1934; Davidsohn and Walker, 1935). In 1935 Davidsohn amended his technique slightly, and in 1937, after there had been further work done on the effect of adsorption on these antibodies by Stuart, Griffin, Fulton, and Anderson (1936), by Stuart, Welch, Cunningham, and Burgess (1936), and by others, he suggested a preliminary adsorption of the sera with various antigens before titration with yet another adaptation of his method. Finally, Barrett (1941) described the technique of absorption and titration which is now widely used.

Attempts have been made to shorten the test by Butt and Foord (1935), Straus (1936), Gleeson-White (1941), and Kaufman (1944), and recently Moloney and Malzone (1949) described a simple slide test which merits further attention. In this test 0.1 ml. of defibrinated sheep's red cells are added to 0.2 ml. of the serum to be tested on a tile, inactivation not being necessary. The test is considered positive if there is gross clumping of the cells in 30 to 60 seconds. The authors state that false positive tests may be obtained with cold agglutinins and with Forssman antibodies. The former can in almost all cases be excluded by performing the test at 37° C. and the latter by adsorption with guinea-pig kidney and subsequent titration. This test, because of its simplicity, would appear to have a possible value in screening suspected sera, thus saving some of the time and labour demanded by the full Paul-Bunnell test. With this in view, a number of sera were examined by both the slide test and the Paul-Bunnell test in an attempt to estimate its reliability.

Method

The method of Moloney and Malzone was used with minor modifications. It was found unnecessary to measure accurately cells and serum, drops from a Pasteur pipette being adequate for the purpose. Maloney and Malzone used undiluted sheep's cells, but, even with the recommended ratio of 2 to 1 of serum to cells, rouleaux formation proved to be troublesome, so a suspension of cells in saline was used. The test was now carried out as follows.

To one drop of unheated, undiluted serum on a tile was added one drop of a 10% suspension of sheep's cells. These were well mixed and the tile gently rocked. Clumping of

the cells within 60 seconds was regarded as a positive result. The test was carried out at room temperature.

Results

Two hundred and sixty-nine sera were examined by the slide test followed by a Paul-Bunnell test by the method of Davidsohn (1929), which is in use in this laboratory. Of these 17 were sera from cases of infectious mononucleosis diagnosed as such on both clinical and haematological grounds (Table I). Of the remaining 252 sera some were from normal pregnant women and prospective blood donors whose blood

TABLE I

RESULTS OF SERA OF INFECTIVE MONONUCLEOSIS EXAMINED BY SLIDE AND PAUL-BUNNELL TESTS

Serum No.	Slide Test	Paul-Bunnell Test (maximum titre)
1	+	1 : 1024
2	+	1 : 2048
3	+	1 : 256
4	+	1 : 360
5	+	1 : 512
6	+	1 : 64
7	+	1 : 128
8	+	1 : 1024
9	+	1 : 128
10	+	1 : 512
11	+	1 : 128
12	+	1 : 128
13	+	1 : 128
14	+	1 : 64
15	+	1 : 256
16	+	1 : 512
17	Negative	<1 : 16

TABLE II

RESULTS OF SERA OF VARIOUS CLINICAL CONDITIONS AND NORMALS TESTED BY SLIDE AND PAUL-BUNNELL TESTS

Clinical Condition	Total	Positive Slide Test	Paul-Bunnell Test			
			1 : 8 or less	1 : 16	1 : 32	1 : 64
Normal pregnancy Blood donors Other normals	114	None	75	26	13	None
Syphilis (W.R. positive)	25	,,	20	3	2	,,
Cancer	11	,,	11	None	None	,,
Cirrhosis of liver	4	,,	4	,,	,,	,,
Lymphatic leukaemia	4	,,	4	,,	,,	,,
Lymphosarcoma	2	,,	2	,,	,,	,,
Reticulosarcoma	1	,,	1	,,	,,	,,
Hodgkin's disease	2	,,	2	,,	,,	,,
Generalized lymphadenitis	2	,,	2	,,	,,	,,
Sarcoidosis	1	,,	1	,,	,,	,,
Banti's syndrome	2	,,	2	,,	,,	,,
Haemolytic anaemia	4	,,	2	1	1	,,
Rh antibodies	1	,,	1	None	None	,,
Miscellaneous	79	,,	59	9	11	,,
Total	252	,,	186	39	27	,,

had been sent to the laboratory for Wassermann testing. The remainder were obtained from patients in the wards of the hospital, and represent a wide variety of clinical conditions some of which are detailed in Table II.

Considering a titre of 1 : 64 to be a positive Paul-Bunnell test (Bunnell, 1933), it will be seen that, in all cases where the slide test was positive, a positive Paul-Bunnell test was also recorded. The case of infectious mononucleosis which gave a negative result by the slide test proved to have a negative Paul-Bunnell test. In no case where the slide test was negative did the titre of antibodies exceed 1 : 32. It would appear that as the antibody titre approaches 1 : 64 so the slide test becomes positive. There does not appear to be any correlation between the degree of clumping of the cells and the antibody titre. Moloney and Malzone reported false positive tests in cases of cirrhosis of the liver and in haemolytic syndromes. This was not found in similar cases examined in the present series.

Summary

Attention is drawn to a slide test for heterophile antibodies in infectious mononucleosis, and some minor modifications are suggested.

In 16 sera which gave positive Paul-Bunnell tests the slide test was also positive.

Two hundred and fifty-two sera from normal subjects and from cases other than infectious mononucleosis were tested and found to have both negative slide tests and negative Paul-Bunnell tests.

It is suggested that the procedure described is a quick and easy test which could be used to screen sera sent to the laboratory for Paul-Bunnell testing. A positive slide test would indicate that the full Paul-Bunnell test should be carried out.

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A PRELIMINARY REPORT ON A MODIFIED FRIEDMAN PREGNANCY TEST

BY

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The Friedman (1939) test for pregnancy has the minor disadvantages that skill is required for the intravenous injection into the ear vein of the rabbit and that some 3% (Albert, 1949; Evans and Krajian, 1944) of urine samples are toxic to the rabbit if injected intravenously. Ducey (1950) overcame some of these difficulties by injecting urine subcutaneously in doses of 20 to 30 ml. in the loose skin between the scapulae.

Using this subcutaneous technique the large, subcutaneous bulge of injected urine, and the not inconsiderable amount of squirming of the test animal while the urine was being injected, were striking disadvantages. Toxic manifestations were not unknown with the subcutaneous technique, especially if the urine was old and somewhat contaminated.

Friedman and Lapham (1931) found that by doubling the minimum effective dose they could produce strongly positive results, i.e., more than six corpora haemorrhagica in both ovaries. Ducey (1950) found, too, that the minimum effective dose by subcutaneous injection was twice that needed for positive results by intravenous injection.

Scott (1940) adsorbed gonadotropins on to kaolin at *p*H 4, and found on elution with sodium hydroxide that most if not all the toxic substances in urine remained adsorbed on the kaolin. This method, too, concentrates the gonadotropins, the concentration depending on the amount of eluent used.

To try to obviate these difficulties the following procedure was evolved.

Modified Friedman Method

The gonadotropins in 100 ml. of urine were adsorbed on kaolin at *p*H 4 after the method of Scott. The gonadotropins were eluted with sodium hydroxide, and half of the neutralized extract was injected subcutaneously into each of two rabbits. With the needle still in position 1 mg. of hyaluronidase was injected from another syringe. The hyaluronidase was prepared from bull semen after the method of Gaisford and Evans (1949). After the injection of the hyaluronidase the animals soon stopped struggling and the subcutaneous bulge rapidly disappeared. No toxic reactions were noted in more than 150 tests.

A noteworthy feature was the fact that 79% of known positive urines were unequivocally positive at 24 hours, against 68% positive by the intravenous route and 59% positive by the subcutaneous route. This is presumably due to the higher

concentration of gonadotropins and the more rapid absorption due to the hyaluronidase. The Friedman test should still be read as a routine at 48 hours before a negative report is issued.

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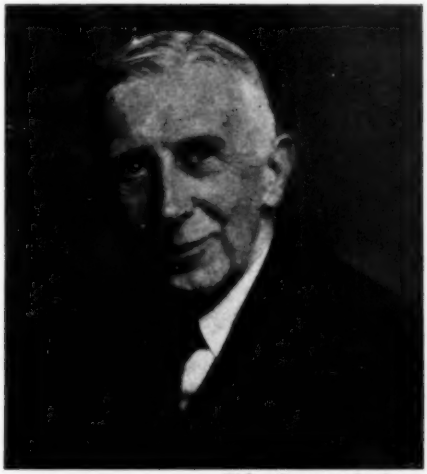
OBITUARY

SIR PHILIP PANTON



Walter Stoneman, London.

PHILIP NOEL PANTON was born in 1877 at Wareham, in Dorset, the son of James Panton and grandson of Frith the Victorian artist. He was educated at Harrow and was the first boy in this school to study science, a fact which he always recollected with interest. At school with him were two great national figures—Winston Churchill and L. S. Amery—and it is interesting to note that a housemaster at that time told Panton that L. S. Amery was far the cleverer boy and would be a future Prime Minister of England.

After leaving Harrow he proceeded to Trinity College, Cambridge, and having decided to study medicine he did his clinical work at St. Thomas's Hospital. He qualified with the Cambridge M.B. degree in 1903 and held house appointments at Addenbrooke's Hospital. He was responsible for initiating the use of autoclaved dressings there, using improvised metal containers. It was at this time that he became interested in cross-infection in hospital wards, a study which he continued when he returned to St. Thomas's as Louis Jenner research scholar under Dudgeon, whom he always described as the first clinical pathologist in London. The subject of cross-infection formed the basis for his Cambridge thesis for the M.D. degree, but a regrettable dispute with Clifford Allbutt led to his refusing to proceed to the degree although the thesis had been accepted.

After being a Gilson research scholar of the Society of Apothecaries Panton was appointed assistant director of the clinical laboratories at St. Thomas's Hospital, a post he resigned on being appointed in 1908 as chemical pathologist to the London

Hospital. He soon had the name of the post altered to that of "clinical" pathologist, and it was in this position that he remained until he resigned from the London Hospital staff in 1946.

In the years before 1914 he rapidly established himself as a reliable consultant and built up a large private practice in pathology. At this time he wrote the first edition of his book *Clinical Pathology*.

During the first world war he carried out research on T.N.T. poisoning, and in 1918 took a commission in the R.A.M.C. but only held it for a very short time.

It was in the two decades of the inter-war years that he did most for the laboratory services of the London Hospital. He appreciated that to produce their best work research workers must be freed of all routine duties but nevertheless should have full access to the material passing through the routine departments. He enlisted the financial aid of the Rev. M. S. Courtauld, and the Freedom Research Fellowships were established; Howard Florey and S. P. Bedson were the first Fellows.

Panton himself was not a great research worker, although he stimulated others to take up research and provided them with facilities to work. He wrote papers on pernicious anaemia, gastric acidity, lymphatic leukaemia, staphylococcal toxins, and in 1939 he published a review of all the known cancer tests. He was a great reader of medical literature and each day spent at least two hours in reading. This enabled him to advise with accuracy on the diagnosis and management of obscure cases, and until he finally retired he was well abreast of the new techniques.

His undergraduate teaching was not very successful, mainly because his conversational tone was difficult to follow except for those in the front rows of his audience, but he was a first-class director of the studies of postgraduate students and proved to be an excellent examiner; he particularly enjoyed examining at Oxford for the M.B. degree.

In 1938 he began to prepare for the possibility of war, and with W. W. C. Topley compiled a record of all pathologists and medical laboratory technicians. This index subsequently proved to be of great value, especially in fitting suitable people into posts in the Services. He was a sector pathologist in the E.M.S. in 1939, but was asked to take up the new post of consultant in pathology at the Ministry of Health in 1940.

For the next seven years he devoted himself to two objects; first to direct a blood transfusion service for the whole country, and secondly to establish a well-organized hospital laboratory service not only for the new emergency hospitals but also one capable of balanced expansion after the war. The development of the transfusion service was of the greatest urgency, and Panton devoted much time to seeing that the regional blood transfusion directors were the best available and that they were given a very free hand. He was also responsible for seeing that the Leeds centre was built up to act as an adjunct to the Army Transfusion Service, and the success of this co-operation gave him great pleasure.

Throughout the war he travelled to every important hospital laboratory in England and Wales, helping the pathologists, arranging for meetings of regional pathologists, and smoothing away local difficulties standing in the path of expansion. He was a good judge of character, and, although many of the young pathologists who had to take charge of laboratories were inevitably woefully lacking in experience, hospital

staffs who were reluctant to welcome a visiting pathologist often complained later if expansion of their laboratory service did not seem to be rapid enough.

In committees at the Ministry he was at his best—tactful, clear-sighted, and so patently honest that he won over Civil Servants to his point of view with the minimum of effort. He never lost sight of the ultimate objective, a first-class hospital laboratory service with pathologists holding equal status with their clinical colleagues. He appreciated the difference between public health bacteriology and hospital bacteriology, and cordially co-operated with Professor G. S. Wilson when the Emergency Public Health laboratory service was being built up. As a member of the Services Committee he advised on the calling up of pathologists for the armed Forces, and his advice was rarely set aside.

Pathologists learned to come to him for advice; he was friendly, hospitable, informed, and always helpful. He felt that a whole-time salaried service was inevitable in hospital pathology, and in spite of the years he had spent in consultant practice he doubted if private practice in pathology would continue.

In 1946 he was rewarded for his war services with a knighthood which he accepted partly, and characteristically, because he said it would help to impress lay committees when he was trying to persuade them to set up adequate laboratory services in their hospitals. In 1946 he lectured in Prague on behalf of the British Council.

After 1948 his work at the Ministry decreased and he was able to spend more time in his delightful garden, his water-garden being his particular joy. Many pathologists will remember with pleasure his informal hospitality at Bourne End. After a very short retirement he had his first coronary thrombosis, but recovered well only to suffer another attack later. He died on December 27, 1950.

It should not be forgotten that the enhanced status of hospital pathologists, and the framework of an adequate laboratory service for the whole country, are largely due to Panton's tireless efforts. Others might have done what he did but none would have done it so well.—H. B. M.

STEPHEN ROODHOUSE GLOYNE

Everyone who had the privilege of knowing and working with Stephen Gloyne was impressed by the essential goodness of his character. It was for this reason that those who had his friendship would repeatedly turn to him for counsel and advice on personal as well as professional matters. His instinctive sympathy and appreciation of problems put to him enabled him to give a judgment that was invariably helpful and stimulating.

Professionally Gloyne was in a unique position for many years as the doyen of chest pathology. After a thorough preliminary training in all branches of pathology he came to the London Chest Hospital, which was his headquarters until he retired. With this position he was content, though in his earlier days he had opportunities for taking up posts which would have led to higher academic rewards. He made many contributions on the morbid anatomy of lung disease, notably silicosis and tuberculosis, and correlated these with the clinical and social aspects of disease. Possibly his most popular lectures and writings were connected with the pathenogenesis of tuberculosis, and his presidency of the Tuberculosis Association was notable for the broad philosophical outlook of the discussions.

During the war a bomb completely destroyed the laboratory that had been built for him, and with it all the records and specimens collected over many years were lost. His highly prized microscope, purchased instead of a motor car, was wrecked, though some of the lenses were salvaged. Gloyne's reaction to the tragedy was characteristic: he said little and, as soon as he could, started off in a temporary building to found his laboratory and specimens again. By this time he was not far off his retiring age, and, though physically as active as ever, was not a fit man. He left a full and busy pathological department undertaking much more work than before the war. His all too short years of retirement were happily occupied at Oxford, where under the roof of the Department of Social Medicine he continued his studies with silicosis up to the time of his death. Many of his former colleagues visited him in Oxford, bringing as of old their problems, some technical and some personal, since he was not a man to be forgotten.

In addition to his work at the London Chest Hospital he was consulting pathologist for many years to the King Edward VII Sanatorium at Midhurst, and also worked for the Silicosis Board.

Gloyne was a prominent member of the Society of Friends, and had a country home in Buckinghamshire not far from Penn and Jordans. He leaves a widow; a son, also a pathologist; and a daughter, an almoner. His epitaph might simply be: " He was a good neighbour."

J. clin. Path. (1951), **4**, 113.

ASSOCIATION OF CLINICAL PATHOLOGISTS: 45th SCIENTIFIC MEETING

The forty-fifth scientific meeting of the Association of Clinical Pathologists was held at the Westminster Medical School, London, from January 25 to 27, 1951, under the Presidency of Professor Sir Lionel Whitby, C.V.O., M.C., M.D., F.R.C.P.

At the first session, for which the President took the chair, four papers were read. Summaries of these follow.

Amidopyrin Agranulocytosis

Dr. GEORGE DISCOMBE said that amidopyrin agranulocytosis was commonly caused by prescribing amidopyrin in a proprietary mixture, the prescriber being ignorant of or forgetting its presence. Four fatal cases had been encountered. In three, sulphapyridine was at first thought to be responsible, but amidopyrin had been taken at the same time. It seems probable that the action on the bone marrow of sulphonamides and amidopyrin is synergic.

Many new drugs containing the benzamine group are coming into use. Most drugs containing this structure can cause agranulocytosis, and the possibility of synergism between amidopyrin and, for example, phenergan is alarming. In Denmark, the distribution of proprietary mixtures containing amidopyrin is forbidden. It seems probable that the same action should be taken in Britain.

The Eosinophil and Other Leucocyte Changes in Burns

Dr. SEVITT reported a study of the quantitative changes in the eosinophil, lymphocyte, and neutrophil counts in 26 patients suffering from burns involving from 0.1 to 80% of the body area. Soon after burning the eosinophil count falls and reaches low or zero values in a few hours. This is often accompanied by a lymphocytopenia and usually by a neutrophil leucocytosis (primary wave). The eosinopenia, which persists from a half to seven days, is of longer duration in those more extensively burned, and there is a statistical correlation between the percentage of skin area burned and the duration of eosinopenia. The significance of these facts was discussed in the light of the known biological and metabolic effects of burning and of the recent knowledge about the effects of ACTH and cortisone. Dr. Sevitt concluded that eosinopenia was a sensitive index of adrenocortical activity in burns. Other leucocyte changes described were the onset of eosinophilia following the eosinopenic stage in some patients, a later or delayed eosinophilia being probably due to allergy and a secondary neutrophil wave.

Fallacies in the Platelet Count due to the Use of Cresyl Blue

Dr. W. K. TAYLOR said that platelet counts in cases of haemolytic anaemia using Lempert's method (*Lancet*, 1935, **1**, 151) were found to be unduly raised. He recommended that Lempert's urea citrate mixture without the addition of dye should be generally used for platelet counts. If the field is not too bright, counting is quite simple.

Factors Controlling Normoblastic and Megaloblastic Erythropoiesis

Dr. L. G. LAJTHA said that from work with bone marrow cultures evidence was presented for the presence of an inhibitory factor in the serum and cerebrospinal fluid of patients with pernicious anaemia. This factor inhibits the ripening of megaloblasts into normoblasts and produces megaloblasts from normoblasts *in vitro*. Folic acid overcomes the action of the inhibitory factor *in vitro* and so does folinic acid (citrovorum factor). Crystalline vitamin B_{12} has no such effect.

The inter-relationship of factors controlling normoblastic and megaloblastic erythropoiesis was discussed.

For the second session the Chairman was Brigadier A. Sachs, M.D., M.Sc. Summaries follow of the papers read at that session.

The Interpretation of Biopsies in Rectal Disease

Dr. C. DUKES discussed the interpretation of biopsies in rectal diseases, and pointed out that the value of the pathological report depended very much on the type of material obtained. The co-operation of surgeon and pathologist, and the experience of both, greatly enhanced the value of the biopsy report. A diagnosis of malignancy was seldom made without evidence of infiltration, and therefore some mucosa should be included in the biopsy. In some cases of well-differentiated tumours without infiltration a diagnosis of malignancy might not be possible. Slides of 30 cases were shown, demonstrating the pitfalls in diagnosis and rare forms of tumour formation. In the practice of his hospital it was now unthinkable to remove a tumour and do a colostomy without a prior biopsy.

Kaposi's Varicelliform Eruption

Drs. A. DICK and N. R. GRIST (Glasgow) described ten cases of Kaposi's varicelliform eruption which appeared at the time of the widespread vaccination occasioned by an outbreak of smallpox in Glasgow. Of these ten patients four were unrelated, two were members of the same household, and four were infected while in hospital. No case had been vaccinated previously and all had been in contact with recently vaccinated subjects and had suffered from previous skin damage. Coloured illustrations of several of the patients were shown.

Fairly complete pathological investigations were undertaken in five cases. The vesicle fluid showed numerous elementary bodies, and cultivation of this fluid or of crusts yielded a coagulase-positive *Staphylococcus pyogenes aureus* in all, while in three the infection was mixed. After suitable treatment the material from crusts or smears was used to inoculate the chorio-allantoic membrane of 11-day chick embryos. The vaccinia virus was isolated from two cases and material sent to Dr. R. H. A. Swain, of Edinburgh, who confirmed the isolation of vaccinia virus from these and from two additional patients. The mode of infection was discussed and evidence was put forward to suggest that the incriminating virus (vaccinia in the present series) is inoculated directly into previously damaged areas of skin.

It seems worth mentioning that two patients appeared to benefit by aureomycin, and a further trial of this drug is indicated.

Cylindrical-celled Carcinoma Presenting as Nasal Polypi

Dr. H. A. LUCAS briefly considered the literature, especially Nils Ringertz's record of 27 cases. He described the clinical and morbid anatomical features of these growths, and their relationship to papillomata. Lantern slides of the histological features, a

diagram illustrating the manner of growth, with tables of age and sex incidence of the cases compared with those in the literature, were shown. Conclusions were drawn as to the nature of these neoplasms, and the importance stressed of complete histological examination of all material obtained by biopsy.

The Misuse of the Term "Adamantinoma"

Dr. K. S. THOMPSON and Professor E. B. MANLEY briefly described normal tooth development as a down growth from the ordinary squamous epithelium from the crests of maxilla and mandible, and drew particular attention to the cytological difference between the cells of the internal dental (enamel) epithelium and the ameloblasts.

They considered that many tumours, such as those in the tibia and pituitary, were wrongly called adamantinomas owing to the oedematous prickle cells superficially resembling stellate reticulum, and they insisted that the diagnosis of adamantinoma should rest on the developmental cell, the cells of the internal dental epithelium.

As ameloblasts are only present with enamel they objected to the application of the term "ameloblastoma" to these tumours, preferring Thoma's "adamantoblastoma," although they felt that long usage made the term "adamantinoma" preferable.

On the afternoon of January 26 the President, Sir Lionel Whitby, gave the presidential address, which will be published in the May issue of the *Journal*. Dr. S. C. Dyke, D.M., F.R.C.P., was in the chair, and Dr. Norah Schuster moved a vote of thanks to the speaker.

During the second day of the meeting seven papers were read. For the first session Professor R. J. V. Pulvertaft, O.B.E., M.D., F.R.C.P., was the Chairman. Sir Henry Cohen read a paper on "The Limitations of Laboratory Investigations."

Accumulation of Penicillin in Inflamed Tissues

Dr. J. UNGAR gave the results of experiments on the distribution of penicillin in infected lung tissue and blood. Bronchopneumonia was produced in mice and guinea-pigs by intranasal instillation of a virulent suspension of *H. pertussis*. When different types of penicillin (aqueous solutions of penicillin G or suspensions) were injected into the infected animals, the penicillin could be detected in their lungs in considerably higher concentrations and for a longer period than in the lungs of normal control animals. The peak of penicillin levels in the inflamed lungs was significantly higher than that of the levels in the blood.

The tissue levels of penicillin depend on the type of penicillin injected (water-soluble or insoluble) and the degree of infection in the lungs; the heavier the infection, estimated by the extent of lung consolidation, the higher are the levels of penicillin in the lung, and they are also detectable there for a longer period than in blood. This finding adds further evidence for the efficacy of single massive doses of penicillin in the treatment of acute infections.

The Diagnosis of Weil's Disease and Canicola Fever with Special Reference to their Atypical Forms

Dr. J. C. BROOM described the customary methods for the laboratory diagnosis of leptospirosis, which are isolation of the organism and the demonstration of anti-

bodies, but in neither case can the diagnosis be established early in the disease. With appropriate techniques it is often possible to see leptospires during the first week in wet preparations of blood, but the difficulty of distinguishing them from artifacts may lead to errors in identification.

It would obviously be helpful if tests could be developed which would enable an early diagnosis to be made with reasonable certainty. A few observations on different aspects of leptospirosis have been made by various workers which, if confirmed and extended, might serve this purpose. It has been found that, in the early days of the illness, (1) the erythrocyte sedimentation rate is greatly increased; (2) the cerebrospinal fluid contains an increased number of cells, even in cases which show no symptoms of meningitis; (3) in the meningeal form of canicola fever the glucose content of the c.s.f. is increased; (4) biopsy specimens of gastrocnemius muscle show the characteristic degenerative changes in single muscle fibres or groups of fibres; (5) biopsy specimens of liver show the characteristic loss of cord structure and also signs of regeneration.

The interpretation of the results of agglutination tests may occasionally present difficulties. Sometimes the antibody titre for the infecting species is, at first, much lower than the corresponding titre for some heterologous species. This may lead to complications, if a hitherto unrecorded species appears to be implicated, as occurred in two instances about 18 months ago. Even such anomalies can, however, be useful on occasion. In a recent case the presence of heterologous antibodies helped to establish the diagnosis of Weil's disease, in a patient who died while being treated with large doses of leptospiral antiserum, and showing only a low agglutination titre to *Leptospira icterohaemorrhagiae*.

Observations on the Epidemiology of Leptospirosis

Dr. J. M. ALSTON said that the illnesses and deaths in the British Isles known to be due to *L. icterohaemorrhagiae* (Weil's disease) were roughly half those due to typhoid fever. The causal organism is excreted in human surroundings in great profusion by rats and, less, by dogs. Leptospiras do not survive long after excretion and that is probably why the disease is limited to those people who, usually by occupation, are frequently or heavily contaminated by rats' urine. In conditions of equal exposure the sexes are equally liable to infection, but only 13% of nearly 1,000 instances in this country were in women or girls. There is a seasonal increase of the disease in the autumn.

The mortality rate for 1949 and 1950 was 12% of observed cases. Death does not occur without obvious jaundice, and nephritis is the most lethal feature. The mortality rate is higher with increasing age. In the non-fatal meningitic form of the disease, the glucose content of the cerebrospinal fluid is normal; infection during bathing tends to lead to meningitis. Reduction of case-mortality requires early diagnosis so that penicillin and antiserum can be used to kill the leptospira before hepatitis and nephritis are severe.

The known incidence of infection by *L. canicola* in the British Isles in 1949 and 1950 is 25 and 33 cases. The source of infection is the urine of dogs which are liable to a severe acute disease which may cause death or chronic nephritis. Human infection is domestic rather than occupational, and the illness in human beings is mild with or without meningitis and is very rarely fatal. Incidence is much more in the second half of the year. The incubation period and natural routes of infection are not clearly known.

In the British Isles there are species of small rodents which are known on the Continent to be carriers of other pathogenic species of leptospiras, not yet found in this country.

Iron Metabolism in Normal Pregnancy and Iron Deficiency Anaemias of Pregnancy and after Iron Administration

Drs. S. VENTURA and A. KLOPPER considered the changes during pregnancy of the serum iron, the iron-binding capacity of the serum proteins, the serum copper level, and the free erythrocyte protoporphyrin. It was shown that the serum iron falls significantly in late pregnancy and that the concentration of the other constituents is increased. The changes in normal pregnancy are not nearly so gross as those demonstrated for the hypochromic anaemias of pregnancy, but they tend to show that the drain of iron during pregnancy causes a state of latent iron deficiency. This was demonstrated further by plotting serum iron curves after oral and intravenous iron administration showing that during pregnancy the intestinal mechanism of iron absorption is orientated toward a maximal transfer of iron to the blood stream, and that, when this mechanism is short-circuited by the intravenous injection of iron, the serum of the pregnant woman still shows enhanced power and speed in dealing with the influx of iron.

Atypical Flocculation Tests in Myelomatosis

Drs. J. H. O. EARLE, N. H. MARTIN, and Professor N. F. MACLAGAN contributed a case history of myelomatosis and the flocculation tests devised for it.

A man aged 59 presented with mild malaise, and was found to have hepatomegaly as the only abnormal physical sign. Puncture biopsy of the liver revealed the presence of amyloidosis for which no obvious cause existed. Liver function tests gave the following results:

Serum bilirubin ..	0.3	mg. per 100 ml.
Serum cholesterol	189	,, ,, ,, ,,
N.P.N.	46	,, ,, ,, ,,
Serum albumin ..	3.7	g. ,, ,, ,,
Serum globulin ..	5.6	,, ,, ,, ,,
Total serum protein	9.3	,, ,, ,, ,,

Serum alkaline phosphatase 21 units. Thymol turbidity 1 unit, thymol flocculation negative, serum colloidal gold negative, $ZnSO_4$ test (Kunkel) 28 units, $(NH_4)_2SO_4$ test (Popper) 21 units.

In view of the unusual flocculation tests electrophoresis of the serum was undertaken with the following results:

Albumin 32%, α globulin 5.0%, β globulin 18.5%, γ_1 globulin 40%, γ globulin 4.0% of total protein.

The γ globulin showed spontaneous precipitation during electrophoresis. The protein picture as a whole suggested a diagnosis of plasmacytoma which was confirmed by radiology of the skeleton where minimal lesions were seen in the skull, mandible, femur, and other bones.

The peculiar dissociation between the various flocculation tests and globulin estimations in this case led to a search of the records at St. George's and Westminster Hospitals and to some further tests on sera from the former hospital. Of the 13 cases of plasmacytoma in which the results were available, 11 showed similar results. It is suggested that the finding of dissociated flocculation tests is a valuable diagnostic aid in plasmacytoma.

Potassium Deficiency States

Dr. MCGOWAN discussed biochemical changes in potassium deficiency states. The main clinical evidence of potassium deficiency was (1) muscle weakness, leading to paralysis; (2) prostration, anorexia, and paralytic ileus; (3) alkalosis, resistant to sodium chloride; (4) E.C.G. changes.

He demonstrated particularly the shift from intracellular to extracellular potassium, with the balance maintained by excretion in the urine and gastro-intestinal secretions, and pointed out that potassium should not be given unless renal function was satisfactory.

The Thigh-Neck Clearance: A Simplified Radioactive Test of Thyroid Function

Dr. J. B. FOOTE and Professor N. F. MACLAGAN presented a paper on their method for estimating thyroid function.

A preliminary report was made to the Society for Endocrinology in December, 1950, since when a further 35 subjects have been tested. The method consists of making alternating neck and thigh counts with a Geiger counter after oral administration in the fasting state of approximately 30 μC. of I^{131} with 20 μg. carrier (expressed as KI). The thigh-neck clearance is calculated by dividing the rate of increase of neck counting expressed as the increase of counts per hour by the thigh count measured during the same period. The measurements are ordinarily made over the period extending from $\frac{1}{4}$ to $1\frac{1}{4}$ hours after I^{131} administration. The patients are restricted to an iodine-poor diet for the three days preceding the test. A special collar and thigh applicator are used to ensure the return of the counter to the same positions for each count. The test occupies approximately one and a half hours, of which about one hour is spent in counting.

The thigh-neck clearance is obtained in arbitrary but reproducible units which vary only with the geometrical constants of the shielding apparatus used. Normal values (23 subjects) were from 1 to 9. Twelve thyrotoxic patients gave values from 24 to 72 and six cases of myxoedema gave zero values. Sixteen cases of non-toxic goitre gave results ranging from 1.5 to 11.5. This procedure has been compared with the plasma iodide clearance (Myant, Pochin, and Goldie, 1949, and Pochin, 1950), and with the neck-thigh ratio at one hour after oral dosage. It is technically simpler than the plasma iodide clearance and is superior to the neck-thigh ratio in the diagnosis of myxoedema.

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The meeting ended with a symposium on "The Manifestations of Anoxia," when the Chairman was Dr. Magnus Haines, M.D. The principal speakers were H. E. Holling (London), H. C. Dalziel (Barnet), C. K. Simpson (London), F. E. Camps (London), and R. Jarman (London).

During the meeting demonstrations were on view at the Royal Army Medical College, Millbank. Two films, *A Hospital Syringe Service* and (*a*) *Kidney Function in Health*, (*b*) *Kidney Function in Disease*, were also shown at the Royal Army Medical College.

The annual dinner was held at Claridges on January 27. The President was in the chair. The toast of the Association was proposed by Dr. Traill (Aberdeen). The guests included Sir Henry Dale, O.M., G.B.E., F.R.S., Sir Henry Cohen, and Dr. Jaumain (Brussels).

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ABSTRACTS

This section of the JOURNAL *is published in collaboration with the two abstracting journals, Abstracts of World Medicine, and Abstracts of World Surgery, Obstetrics and Gynaecology, published by the British Medical Association. In this* JOURNAL *some of the more important articles on subjects of interest to clinical pathologists are selected for abstract, and these are classified into four sections: bacteriology; biochemistry; haematology; and morbid anatomy and histology.*

BACTERIOLOGY

The Effective Concentrations of Penicillin *in vitro* and *in vivo* for Streptococci, Pneumococci, and *Treponema pallidum*. EAGLE, H., FLEISCHMAN, R., and MUSSELMAN, A. D. (1950). *J. Bact.*, **59**, 625.

The minimal effective concentration of procaine penicillin in the serum of mice sufficient to abort clinical infection following intramuscular injection of group A streptococci, group B streptococci, and types I and III pneumococci were found to be 0.012, 0.08, 0.08, 0.05, and 0.05 mg. per ml. In mice receiving group B streptococci by intramuscular, subcutaneous, or intratesticular injection and direct injection into the lung, the values were 0.12, 0.1, 0.07, and 0.1 mg. per 1 ml. respectively. In rabbits infected by the intratesticular injection of *Treponema pallidum*, the values varied from 0.005 to 0.01 mg. per ml. Approximately the same values were obtained with aqueous penicillin.

In *in vitro* tests, the levels necessary to kill the same organisms were regularly one-half to one-fifth of those given above. The discrepancy between the *in vivo* and *in vitro* values is accounted for partly by the fact that about half the penicillin in serum is bound to the serum proteins and partly because the concentration of penicillin in the tissues is probably lower than that in the serum. For these reasons, it is probable that the effective concentration of penicillin at the actual focus of infection is of the same order of magnitude as that which obtains *in vitro*.

Thus for the organisms studied, penicillin is effective *in vivo*, so long as its serum concentration is two to five times the minimal bactericidal level as determined by tests with the serum *in vitro*. *R. Hare.*

Prolongation of Penicillin Blood Level by Implantation of Procaine Penicillin Tablets. HUIDOBRO, F., CROXATTO, R., and LUCHINI, A. (1950). *Proc. Soc. exp. Biol., N.Y.*, **73**, 201.

The authors, working at the Catholic University of Chile, Santiago, found that the implantation of tablets of procaine penicillin (20,000 to 80,000 units per kg. body weight) subcutaneously in rabbits resulted in an effective concentration of penicillin in the blood for 7 days and gave no local reactions. When they were mixed with fat the period was prolonged to 11 days.

Chloromycetin in Surgical Infections. ALTEMEIER, W. A., and GIUSEFFI, J. (1950). *Surg. Gynec. Obstet.*, **90**, 583.

The authors give a brief survey of the main physical and chemical properties of chloramphenicol and include a list of the organisms known to be susceptible to its action. There follows a report on the treatment of 100 surgical patients selected at random. The daily dosage varied from 25 to 100 mg. per kg. body weight, single doses being given at intervals of 4 to 8 hours. There were 23 cases of infection with staphylococci, 4 with aerobic streptococci, 3 with anaerobic

streptococci, 43 with mixed Gram-negative and Gram-positive organisms, 8 with Gram-negative bacilli only, one with *Clostridium welchii*, and a miscellaneous group.

In the staphylococcal group the average duration of treatment was 6.7 days and the average total dose of chloramphenicol 8.6 g. In 21 of the 23 cases the results were classified as " excellent " or " good." Penicillin is still, however, the antibiotic of choice in these infections, although chloramphenicol is of value in penicillin-resistant cases. In the 43 cases of mixed infection the average duration of treatment was 12.44 days and the average total dose of chloramphenicol 17.82 g. " Excellent " or " good " results were obtained in 31 cases, " doubtful " results in 9, and there were 3 failures. [Figures are given for the other types of infection, but the numbers seem to be too small to be of any value.]

Bacterial resistance developed in 11 of the 100 cases, the infection in 5 being due to *Bacillus proteus*. The time taken for resistance to develop varied from 5 to 33 days. [No details are given of the degree of resistance encountered.]

The authors stress that the ordinary fundamental principles of surgical treatment are still as important as ever.

A. W. H. Foxell.

Chloramphenicol in Typhoid Fever. GOOD, R. A., and MACKENZIE, R. D. (1950). *Lancet*, **1**, 611.

An assessment of the action of chloramphenicol (" chloromycetin ") in typhoid fever was made in a group of 13 cases from the Crowthorne epidemic of 1949 (Vi-phage type E_1). The cases were first seen on the 8th to 14th day of the disease; 6 were treated with chloramphenicol and 7 were given general hospital treatment only. Later, relapses in 3 of the control patients were also treated with chloramphenicol. Positive stool cultures were obtained from all cases on admission, and frequent cultures of stools and blood were made during and after treatment. The dosage of drug given was that recommended by Woodward *et al.* (*Ann. intern. Med.*, 1948, **29**, 131), a loading-dose of 4 g. in the first hour being followed by 0.25 g. 2-hourly until the temperature was normal, and thereafter 4-hourly until the end of the 8-day course. The total dosage was 19 to 22 g.

In all treated cases the temperature fell to normal within 3 days; headache, toxaemia, and abdominal discomfort were relieved and the appetite returned. No haemorrhages or perforations occurred, and no toxic effects were observed during administration of the drug. Three of the 6 treated cases relapsed and 2 were given further chloramphenicol treatment with good results; no drug-resistance could be detected in the organisms isolated from these cases.

A single course of treatment had no significant effect upon the rate at which the stools were cleared of typhoid bacilli, but 2 of the patients who relapsed after the first course were rapidly cleared by a second course. In the third case of relapse a chronic carrier state developed and excretion of *Salmonella typhi* in the stools continued throughout the second course of treatment. Relapses in treated cases occurred about the 35th day of the disease, and in controls about the 25th day.

No variations in H, O, or Vi agglutination titres of the sera could be attributed to chloramphenicol treatment.

L. G. Goodwin.

The Effect of Aureomycin on the Bacterial Flora of the Intestinal Tract of Man. A Contribution to Pre-operative Preparation. DEARING, W. H. (1950). *Proc. Mayo Clin.*, **25**, 87.

The following regimen was used in the pre-operative preparation of 148 patients suffering from carcinoma of the colon, colitis, or enteritis (not all of whom were in fact operated upon): (1) low-residue diet and mild saline laxatives; (2) a series of saline enemata 1 to 2 days before operation; and (3) chemotherapy. This last consisted of aureomycin 250 to 750 mg. four times a day by mouth (80 patients), " sulphasuxidine " (succinylsulphathiazole) 4 g. six times in one day and 2 g. six times a day thereafter (32 patients), " sulphathalidine " (phthalylsul-

phathiazole) in the same dosage as sulphauxidine (7 patients), dihydrostreptomycin orally 500 mg. four times daily (9 patients), or a combination of aureomycin and dihydrostreptomycin (20 patients). Medication was continued for varying periods, averaging 3 to 5 days but varying from 1½ to 10 days. A fresh specimen of stool was cultured each morning for aerobes, anaerobes, and (after heating) for spores.

In most cases all organisms initially present except *Proteus* and *Pseudomonas* were removed by the aureomycin. In 7 cases the growth of *Bacterium coli* was diminished but not eliminated, and *Streptococcus faecalis* persisted in 4 cases. The sulphonamides and dihydrostreptomycin were much less effective, although the latter combined with aureomycin gave good results when the type of case is taken into account. Bacteroides were not readily detected by the techniques used, but a separate investigation of 5 patients showed them to be removed by aureomycin therapy. Sterilization of the intestinal tract was hindered in cases of pre-existing perforation with abscesses, fistulae, or obstruction, which probably interfered with access of the aureomycin to the organisms.

The authors recommend the administration of aureomycin orally, 750 mg. four times daily for 3 to 3½ days, for the preparation of patients for intestinal operations.

Aureomycin Treatment of Infantile Diarrhoea and Vomiting. MAGNUSSON, J. H., LAURELL, G., FRISELL, E., and WERNER, B. (1950). *Brit. med. J.*, **1**, 1398.

The investigations and treatment of two epidemics of infantile diarrhoea and vomiting in the Sachs Hospital for Children are described. The first occurred in 20 infants during July–September, the second in 14 infants during September–November. All the infants in the first epidemic and 10 of the 14 infants in the second became affected during their stay in hospital.

Bact. coli neapolitanum (B.C.N.) was demonstrated in 19 cases in the first epidemic and in all in the second. The organism was found in the respiratory tract of 15 cases, in the throat only in 3, in the nose and throat in 12. Out of a control series of 40 children in hospital at the same time, but without diarrhoea or vomiting, B.C.N. was found in the stools of one only.

The incubation period was from 4 to 17 days, most commonly between 8 and 12 days. The commonest symptom was diarrhoea, usually accompanied by vomiting. In the first epidemic 8 cases were mild and 12 moderately severe or severe; 5 children died. In the second epidemic, 2 cases were mild and 11 severe; no child died. All the infants were less than 1 year of age and 22 less than 1 month old; 12 were premature, 7 in the first epidemic and 5 in the second.

Penicillin, sulphonamides, and streptomycin were without effect. Aureomycin was given orally, 25 mg. six times a day, to 8 cases in the second epidemic after B.C.N. was found to be sensitive to this antibiotic; the micro-organism's resistance varied between 0.16 and 0.6 μg. per ml., in thioglycollate broth. The stools became firmer and the vomiting ceased, usually in 24 hours; by the 3rd to the 5th day the stools were normal and there was a satisfactory gain in weight.

Aureomycin in Soft Tissue Infections. LOGAN, M. A., METZGER, W. I., WRIGHT, L. T., PRIGOT, A., and ROBINSON, E. A. (1950). *Amer. J. Surg.*, **79**, 229.

A series of 82 cases, which included cases of most of the surgical infections commonly encountered, was treated with aureomycin. In mild cases a dosage of 250 mg. orally three times a day was employed. In severe cases an initial dose of 300 mg. in 500 ml. of 5% glucose in distilled water was given intravenously and this was repeated 12 hours later, after which oral administration was begun. Occasional nausea and vomiting were the only toxic reactions to oral administration. In 15 to 20% of cases in which the intravenous route was used a chemical phlebitis developed. The use of a leucine buffer as a diluent instead of distilled water reduced the frequency of this complication.

The conditions studied included cases of cellulitis, perirectal infection, peripheral ulcer, lymphadenitis, wound infections,

and gas gangrene. The commonest organisms were staphylococci or β-haemolytic streptococci. In most cases one or two species of bacteria were found initially and could still be isolated 3 or 4 days after the institution of aureomycin therapy. In open wounds under treatment, the secondary contaminant organisms commonly found were still present. No evidence was found of the development of drug resistance with prolonged administration.

A wide range of Gram-negative and Gram-positive organisms are sensitive to aureomycin. The level in blood obtained by oral administration is sufficient to control growth of most of these. Clinically results were comparable whether the drug was given intravenously or orally. In the reported cases the dramatic influence of aureomycin on severe and mixed infections is well shown. Cellulitis, for example, treated before necrosis and pus formation, subsided without going on to fluctuation. Out of 4 cases of gas gangrene reported, one elderly arteriosclerotic patient died. Two other patients developed gas gangrene in spite of penicillin therapy. In both of these cases after adequate operation and aureomycin therapy recovery was rapid.

[The satisfactory response of a wide variety of infections to aureomycin is well shown by this paper. If results quoted are supported by further clinical experience, this will unquestionably mean the replacement of the more troublesome penicillin therapy by oral administration of aureomycin.]

J. G. Bonnin.

The Susceptibility *in vitro* of Some Salmonella and Shigella Strains to Chloramphenicol and Aureomycin. ALIN, K., and WAILMARK, G. (1950). *Nord. Med.*, **43**, 663.

A total of 75 strains was tested for sensitivity including *Salmonella typhi*, *Salm. paratyphi B*, *Salm. typhi-murium*, *Salm. bareilly*, and *Salm. montevideo*, and *Shigella sonnei*.

Aureomycin was the most active compound, and inhibited all strains in a concentration of 8 μg. per ml.; chloramphenicol produced complete inhibition in a concentration of 16 μg. per ml., and several strains survived exposure to higher concentrations of streptomycin. The average level of aureomycin in blood is lower than the concentration required for complete inhibition of these strains *in vitro*, whereas that of chloramphenicol is considerably higher. Chloramphenicol is thus to be preferred in the treatment of salmonella infection, although the organisms are more sensitive to aureomycin.

Fatalities in Salmonella Infections. SAPHRA, I. (1950). *Amer. J. med. Sci.*, **220**, 74.

During a period of 10 years ending in April, 1949, nearly 5,000 salmonella cultures were identified at the New York Salmonella Centre. An examination of the records in the 3,279 cases of human infection revealed that 174 fatalities occurred, 26 salmonella types being isolated from these fatal cases. The most virulent types were *Salmonella typhi-murium*, *S. cholerae suis*, *S. oranienburg*, and *S. newport*. The infection was with *S. typhi-murium* in 63 of the fatal cases and with *S. cholerae suis* in 40. The fatality rate in cases of infection with the latter micro-organism was 21.3%; it was seldom isolated from the faeces or from healthy carriers. In most cases the cultures were derived from parenteral sources and from material obtained at necropsy. No deaths were due to *S. paratyphi A*, a type which is rarely found in the United States. Another rare type was *S. havana*, which was originally isolated in 1939 from material from 21 newborn babies in a maternity hospital in Havana. The infection produced the signs and symptoms of acute meningitis and every baby died.

The mortality was about 2% in patients aged between 1 and 50 years, 5.8% in infants, and 15% in elderly patients. In about one-third of the fatal cases occurring in patients aged more than 50 years the infection was a terminal event in diseases such as hypertension, pneumonia, and cancer; as these patients had become debilitated it was possible for salmonellae to pass from the

intestinal tract into the blood stream. Although acute gastro-enteritis was a common cause of death, especially in infants, the clinical manifestations of salmonella infection included meningitis, pneumonia and pleurisy, septicaemia, and bacterial endocarditis. Other manifestations were cholecystitis, appendicitis, peritonitis, and typhoidal syndromes. *A. Garland.*

Clinical Observations on the Use of Terramycin Hydrochloride. KING, E. Q., LEWIS, C. N., WELCH, H., CLARK, E. A., JOHNSON, J. B., LYONS, J. B., SCOTT, R. B., and CORNELY, P. B. (1950). *J. Amer. med. Ass.*, **143**, 1.

Terramycin is an antibiotic isolated from *Streptomyces rimosus*. It is a crystalline substance which forms salts with both acids and bases. This preliminary communication reports the results of the oral administration of terramycin hydrochloride to 30 patients with various infections. In the majority of cases the following dosage schedule was employed: in patients 14 years of age and older, 500 to 750 mg. every 6 hours; in children 9 years of age and younger 500 mg. every 4 hours. Assayable amounts of terramycin were found in the blood and urine for a period of 1 to 5 hours following the administration of 750 mg. by mouth.

Fifteen of the patients had pneumococcal pneumonia or lung abscess and the remainder had bronchopneumonia, urinary infections due to *Aerobactes aerogenes*, *Bacterium coli*, streptococci, *Bacillus pyocyaneus*, or *Staphylococcus aureus*, or bacteraemia due to *Salmonella cholerae-suis*. There were also 2 cases of pertussis. One patient with lobar pneumonia died within 24 hours of admission and in one case treatment was abandoned owing to vomiting: of the remainder, the result in 24 cases is classified as " good " or " excellent " and in 4 as " fair." Six of the 12 children treated developed toxic reactions which included nausea, abdominal pain, vomiting, diarrhoea, and transient erythema, but in only 2 cases had the drug to be stopped. It is concluded that terramycin is a useful antibiotic with a wide bacterial spectrum. *A. W. H. Foxell.*

A Study of the Effect of Neomycin and Other Antibiotics on Bacteria, Viruses, and Protozoa. FELSENFELD, O., VOLINI, I. F., ISHIHARA, S. J., BACHMAN, M. C., and YOUNG, V. M. (1950). *J. Lab. clin. Med.*, **35**, 428.

The action of neomycin was tested on 370 organisms. It showed strong action *in vivo* and *in vitro* against Gram-negative bacilli, streptomycin-sensitive and resistant strains of tubercle bacilli, *Bacillus anthracis*, *Listeria* (*Listerella*) *monocytogenes*, *Proteus* and *Pseudomonas*, and intestinal protozoa. It was effective against *Entamoeba histolytica* and the organism of rickettsialpox. Its action on Gram-positive cocci was variable. Its activity was compared with that of penicillin, streptomycin, chloramphenicol, aureomycin, polymyxin D, bacitracin, and enniatin III. It was more effective than streptomycin against salmonellae and *Vibrio cholerae*, but showed no activity against the viruses of herpes simplex, influenza, equine encephalitis, and lymphocytic choriomeningitis. Its toxicity is lower than that of polymyxin D.

A. W. H. Foxell.

Use of Pituitary Adrenocorticotropic Hormone (ACTH) in Poliomyelitis. CORIELL, L. L., SIEGEL, A. C., COOK, C. D., MURPHY, L., and STOKES, J. (1950). *J. Amer. med. Ass.*, **142**, 1279.

The fact that so many instances of infection with the virus of poliomyelitis are not accompanied by any paralysis has suggested to some that in this disease resistance involves non-specific defence mechanisms and that host resistance may be more important than virulence in determining the outcome in any given case [granted that this statement has any real semantic importance]. For these reasons investigations were carried out to decide whether adrenocorticotrophic hormone (ACTH) given early in the course of poliomyelitis can stimulate effective resistance to the virus.

In a carefully controlled experiment, 35 patients, whose condition was diagnosed as poliomyelitis, received the hormone (dose not stated) while 35 others, selected a

random, were given a placebo. Activity of the hormone was controlled by eosinophil counts, which clearly demonstrated that the desired response was in fact elicited. The result of this investigation proved entirely negative, statistical analysis showing that ACTH is without either beneficial or deleterious effect on the course of poliomyelitis when it is given after the first onset of symptoms. *Jos. B. Ellison.*

HAEMATOLOGY

Effect of Thymine Desoxyriboside (Thymidine) on Human Pernicious Anemia. REISNER, E. H., and WEST, R. (1949). *Proc. Soc. exp. Biol., N.Y.*, **71**, 651.

Thymidine given to patients with pernicious anaemia provoked a slight reticulocytosis but was without effect on the erythrocyte count. Although the replacement ratio of thymidine to vitamin B_{12} is about 300: 1 for the growth of *Lactobacillus acidophilus*, it does not appear to be able to replace vitamin B_{12} in the treatment of pernicious anaemia in man.

Observations of the Hemopoietic Factors in Hog Stomach and Duodenum, and the Treatment of Pernicious Anemia by Orally Administered Vitamin B_{12} in Combination with Extracts of Duodenal Mucosa. BETHELL, F. H., SWENDSIED, M. E., MEYERS, M. C., NELIGH, R. B., and RICHARDS, H. G. (1949). *Univ. Hosp. Bull., Ann Arbor*, **15**, 49.

The vitamin B_{12} content in 2 samples of desiccated hog stomach was assayed using *Lactobacillus lactis Dorner* and found to be 0.23 and 0.31 μg. per g. of material. Daily doses equivalent to 4.6 and 12.4 μg. vitamin B_{12} administered to patients with pernicious anaemia caused satisfactory responses but not if the preparations had been heated to 100° C. for 30 minutes. Evidence was obtained that hog stomach contains intrinsic factor as well as vitamin B_{12}. Neither factor was effective when administered singly by mouth in doses which together caused prompt responses.

Haemolytic Disease of the Newborn due to Anti-A Antibodies. BOORMAN, K. E., DODD, B. E., and TRINICK, R. H. (1949). *Lancet*, **1**, 1088.

The authors describe two cases of haemolytic disease of the newborn and present evidence indicating that this was due to anti-A antibodies. In the first case it was demonstrated that group A, but not group O, erythrocytes were rapidly eliminated from the infant's circulation; in the second (fatal) case anti-A antibodies were recovered from the infant's liver, kidney, and spleen.

Urethane (Ethyl Carbamate) Therapy in Multiple Myeloma. LOGE, J. P., and RUNDLES, R. W. (1949). *Blood*, **4**, 201.

Four patients were treated with doses of 4–6 g. urethane daily for periods up to 2 months. Transient leucopenia developed in three of them. There was some clinical improvement, usually commencing after 2–3 weeks, and two of the patients were able to do light work after 4–6 months. There was also objective improvement; e.g., a rise in the haemoglobin and a reduction in the numbers of abnormal cells in the peripheral blood, a diminution in the number of plasma cells in the marrow, and a reversion towards normal of the serum proteins.

Change in Tendency to Aggregate as an Expression of Disease of Erythrocytes. MLCZOCH, F., WUNDERLY, C., and WUHRMANN, F. (1949). *Acta med. scand.*, **134**, 260.

The erythrocyte sedimentation rate depends on the production of aggregates of erythrocytes, and the rate is increased when such clumps are larger than normal. Factors in the plasma producing clumping include fibrinogen and certain globulins, but the tendency to form aggregates varies in different species of animals.

Erythrocytes were washed several times in normal saline, and after re-suspension in a solution of pectin, a colloid substance, the sedimentation rate was estimated for 2 hours by Westergren's method. Certain variations were noted in different concentrations of pectin, and therefore the ordinary sedimen-

tation rate was estimated also. Erythrocytes from patients with pernicious anaemia showed more rapid sedimentation, but the rate approached the normal in remission. The presence of post-haemorrhagic anaemia did not influence sedimentation. In leukaemia and other disorders of the bone marrow sedimentation in pectin was more rapid, but in a few cases of epidemic hepatitis and of obstructive jaundice it was slower, a unique finding. The authors believe that by their test they can distinguish between disorders of erythrocytes and disorders in which erythrocytes are affected only secondarily.

E. Neumark.

Generalized Blood Platelet Thrombosis. Report of Three Cases with Necropsy Findings. GREEN, M. A., and ROSENTHAL, S. (1949). *J. Mt. Sinai Hosp.*, **16**, 110.

Generalized platelet thrombosis occurs especially in young females and is an acute disease lasting a few weeks from onset to fatal termination. Symptoms of thrombocytopenic purpura—petechiae, ecchymoses, melaena, haematuria—and anaemia occur, but in addition there are arthralgia, headache, nausea, and general malaise. In the course of the illness signs of affection of the central nervous system appear, such as mental changes and focal symptoms—cranial nerve palsies, localized pareses, and aphasia; these neurological signs are transitory and variable. There is a low platelet count and a variable anaemia, sometimes haemolytic in type, and the bone marrow is hyperplastic with a normal count of megakaryocytes. Cerebrospinal fluid is normal. Splenectomy was tried in 2 cases without effect. Microscopical examination of tissues at necropsy reveals numerous thrombi in the terminal arterioles and capillaries of many organs, including the cerebral cortex. These thrombi contain no erythrocytes or white cells and appear to consist of platelets; organization occurs quite early. The thrombi in the cerebral cortex cause little if any parenchymatous change. Fifteen cases have been recorded in the literature and the authors add 3 more. The aetiology remains unknown.

M. C. G. Israëls.

Thrombopathic Thrombocytopenia: Successful Transfusion of Blood Platelets. HIRSCH, E. O., FAVRE-GILLY, J., and DAMESHEK, W. (1950). *Blood*, **5**, 568.

A child 9½ years old had had a splenectomy for haemorrhagic phenomena when 2½ years old. When seen recently he had anaemia, a tendency to haemorrhage, particularly from the nose, a marked diminution in prothrombin consumption, and a prolonged bleeding time even when platelet levels were between 150,000 and 280,000 per c.mm. The platelets were, however, morphologically abnormal, being large, bizarre, lacking in granules, and staining poorly. Similar abnormalities were noticed in the megakaryocytes. The condition is described as one of "thrombopathic thrombocytopenia." Transfusion of stored or citrated blood was without effect. Direct transfusion of blood from a polycythaemic donor, whose platelet count was about eight times the normal, with silicone-lined syringes and no anticoagulant, resulted in the recovery of a detectable proportion of donor platelets from the patient and in improvement in prothrombin consumption and decrease in bleeding time. The authors conclude that their data suggest that the life span of the transfused platelets was 5 to 6 days.

Janet Vaughan.

Thrombocytopenic Purpura: The Prognostic and Therapeutic Value of the Eosinophil Index, an Analysis of 100 Cases. SCHWARTZ, S. O., and KAPLAN, S. R. (1950). *Amer. J. med. Sci.*, **219**, 528.

The authors review the experience of the last 10 years which has led them to attach importance to the finding of a high eosinophilic index in thrombocytopenic purpura. The latter, determined from bone-marrow smears stained by May–Grünwald and Giemsa, is the number of eosinophils per 1,000 neutrophils at the metamyelocyte stage or later. An index above 50 indicates a 50% chance of spontaneous recovery, and a 96% chance of cure by splenectomy. This eosinophilia was not demonstrable in the circulating blood. Splenectomy performed in 18 cases when the index was low resulted in 8 deaths,

and in 2 other cases there was no improvement. *E. T. Ruston.*

Observations on the Coagulation Defect in Thrombocytopenic Purpura. CARR, T. L., and FOWLER, W. M. (1949). *J. Lab. clin. Med.*, **34**, 1227.

The Waugh–Ruddick test for increased blood coagulability (*Canad. med. Ass. J.*, 1944, **50**, 547) was carried out on the blood of 30 normal subjects, the results being in agreement with those of the originators. The test was then carried out on 15 patients suffering from thrombocytopenic purpura, 9 of the cases being of the idiopathic type. It was found that the coagulation time was normal in the absence of heparin, but was markedly increased with even small amounts of heparin and that the blood was easily rendered incoagulable. In 4 cases of non-thrombocytopenic purpura the result of the test was normal. Protamine sulphate and toluidine blue (1 to 4 mg. per kg. body weight) were given to 7 patients with thrombocytopenic purpura and the test then carried out on samples of their blood. In 2 cases there was no demonstrable effect. In one case the coagulation time returned to normal after the injection of protamine sulphate, but this was thought to be due to a spontaneous remission. In the remaining cases the coagulation time was shortened in the tubes containing the lower concentrations of heparin. The test was also carried out in 5 cases of thrombocytopenia before and after splenectomy. In 4 cases the platelet count returned to normal, as did the result of the Waugh–Ruddick test. In the fifth case the platelet count and the test result became normal, but when, 2 months later, cutaneous haemorrhages reappeared and the platelet count fell to its previous low level the coagulation times in the Waugh–Ruddick test were even more prolonged than initially.

The authors conclude that, while the blood of patients with thrombocytopenic purpura is more sensitive to heparin *in vitro* than is normal blood, the coagulation defect is not due to an excess of heparin in the circulating blood since injection of such agents as protamine sulphate and toluidine blue does not remedy the defect, as would be the case were excess of heparin the basis of the changes. *R. B. Lucas.*

Clinical Studies of the Heparin Cofactor. HOLDEN, W. D., COLE, J. W., and DAVIS, J. H. (1949). *Surg. Gynec. Obstet.*, **89**, 20.

An increase in the coagulability of the blood after operation may be an important factor in the causation of post-operative thrombosis. Such an increase would result from a reduction in the quantity of any of the thrombin-inactivating agents present in the blood. Heparin in itself is not an anti-thrombin but requires for its activation the presence in the plasma of a cofactor, the exact nature of which is not known, but which is closely associated with the protein fraction of the plasma. In order to carry out a quantitative estimation of the heparin cofactor in a given blood sample, the serum is heated to 56° C. for 10 minutes, which destroys the thrombin and prothrombin but leaves the cofactor unchanged. After the addition of a known quantity of heparin and of thrombin to the inactivated serum, it is then possible to determine the cofactor activity of the serum by adding fibrinogen and measuring the coagulation time.

The heparin cofactor activity of the blood was estimated in 67 normal fasting subjects and 22 patients undergoing major surgical operations, on whom tests were carried out before, and on alternate days after, operation. In 20 of the patients no thrombosis occurred and the average deviation from normal in cofactor activity of the blood was insignificant. In the other two cases, however, a marked reduction in cofactor activity (5.8 and 15% of normal respectively) was followed by the onset of clinical thrombosis. In a small group of 6 patients with low blood albumin levels, a direct relation was found between blood cofactor activity and blood protein concentration, the former being markedly reduced in all cases and returning to normal in those in which the hypoproteinaemia was eventually relieved.

H. Payling Wright.

Disturbances of Clotting in Acute Leukaemia. Biological and Therapeutic Study. SOULIER, J. P., and DAUSSET, J. (1950). *Sang*, **21**, 602.

This report is based upon a study of the blood-clotting mechanism in 21 cases of acute leukaemia. The authors found a constant reduction in platelet count which, they suggest, is responsible for the prolonged bleeding time, increased capillary fragility, incomplete utilization of prothrombin, failure of clot retraction, and increase in protamine index and prothrombin time in such cases. They could find no evidence of the presence of a heparin-like substance in the serum in any of the 6 cases in which it was sought. All forms of therapy are transient in their effect.

E. T. Ruston.

Refractoriness in Haemophilia to Coagulation-promoting Agents: Whole Blood and Plasma Derivatives. FROMMEYER, W. B., EPSTEIN, R. D., and TAYLOR, F. H. L. (1950). *Blood*, **5**, 401.

In this paper details are given of 22 cases of haemophilia which had been treated with blood and/or Cohn's fraction I (antihaemophilic globulin). Five of these patients were proved to be refractory to treatment, for the usual reduction in coagulation time following transfusions of blood or plasma fraction was not observed. Circulating anticoagulants were demonstrated in their blood, and evidence was obtained that in these cases antibodies had developed against an antihaemophilic substance present in prepared plasma fractions and normal whole blood.

MORBID ANATOMY AND HISTOLOGY

Granulomas of Large Size Caused by Implantation of Talcum (Talcum Sarcoids). GRUENFELD, G. E. (1949). *Arch. Surg., Chicago*, **59**, 917.

The author seeks to shake complacency about the inertness of glove powder introduced at operation by recalling the warnings in the literature of recent years. He adds a personal case report of a rapidly growing tumour developing in the deeper layers of the scar 12 years after appendicectomy. On section it appeared as a large conglomeration of giant-cell tubercles with no round-cell infiltration. Needle-like inclusions in the giant cells were proved by polarized-light study to consist of crystalline silicate known to be present in talcum powder.

This "sarcoid" tumour is the rarest and most severe form of reaction to surgical glove powder, the milder forms of which are also recapitulated by the author. Several theories are advanced, but the exact mechanism of this foreign-body reaction appears not to be known. No dependable substitute for talcum powder has as yet been advanced.

R. P. G. Sandon.

Pathogenesis of Atherosclerosis. DUGUID, J. B. (1949). *Lancet*, **2**, 925.

This discussion of the mechanism of formation of atheroma (atherosclerosis) is partly a resuscitation of Rokitansky's views. The author's thesis, which is based on a wide study, reported elsewhere, of frozen sections of the aorta and arteries, is that the atheromatous plaque is the result of thrombotic accretions on the vessel wall, with subsequent contraction, organization, and compression of the mass. The author believes that the commonly accepted conceptions of the pathogenesis of atheroma originated by Virchow are quite incompatible with the kinetic hydrostatic conditions which are presumed to exist within the arteries during life. He admits, however, that the amount of fatty and lipoid material in the base of the atheromatous plaque cannot all be attributed to the breakdown of the thrombus. The complexity of the relationship between fatty change, ulceration, and thrombosis is discussed and the question left open.

A. C. Lendrum.

Measles Encephalitis. APPELBAUM, E., DOLGOPOL, V. B., and DOLGIN, J. (1949). *Amer. J. Dis. Child.*, **77**, 25.

The problem of acute encephalitis associated with the exanthemata is discussed in relation to 74 cases of measles ence-

phalitis, observed over a 10-year period in one hospital. The chief interest of the study lies in the post-mortem findings in 7 fatal cases. The most pronounced feature in those with the shortest history of neurological symptoms before death (3 days or less) was the presence of scattered areas of cellular infiltration of the adventitia of the veins. When symptoms had been present for 3 days or longer, the better recognized sign of demyelination began to be obvious, accompanied or followed by increase of microglia in response to cell damage. The authors consider the condition primarily inflammatory. Certainly in cases where neurological signs precede or appear with the rash, perivascular infiltration or a haemorrhagic encephalitis is commonest. The nature of the association between the exanthem and the encephalitis remains unsolved.

Sequelae are common. In a follow-up study of 33 cases, only 13 appeared entirely normal. Minor or major signs of cerebral alterations were present in the remainder. In the acute stage, human measles immune serum and human pooled plasma were without effect. *W. G. Wyllie.*

The Pathology of Gaucher's Disease in Early Childhood (with Special Reference to the Neurological Form). GIAMPALMO, A. (1949). *Acta paediatr., Stockh.*, **37**, 6.

The author studied two siblings with Gaucher's disease beginning in the one case in infancy and in the other in early childhood and pursuing a rapidly fatal course. He collected from the literature another 28 cases of Gaucher's disease occurring in the first year of life, and on the basis of these 30 cases he describes the pathological features and the symptomatology of Gaucher's disease at this age. When the disease appears in infancy it differs from the same condition in older children and adults. The differences are that it runs a much more rapid course, death occurring always within the first two years of life, that superficial lymph nodes are usually enlarged, that skin changes are absent and blood changes slight, and that frequently neurological symptoms form a prominent part of the clinical picture.

[The details of the clinical picture, the pathological changes, and the author's views on the pathogenesis of the condition should be read in the original.]

S. A. Doxiadis.

Cystadenoma of the Pancreas. A Report of Two Cases Showing Calcification. HAUKOHL, R. S., and MELAMED, A. (1950). *Amer. J. Roentgenol.*, **63**, 234.

In the two cases reported the tumour occupied the head and tail of the pancreas respectively. Both occurred in women (53 and 75 years) who gave short histories of abdominal symptoms. Calcification has not previously been reported in this type of tumour, according to the author.

Cushing's Syndrome and Thymic Carcinoma. HUBBLE, D. (1949). *Quart. J Med.*, **18**, 133.

A detailed description is given of the symptoms and signs and necropsy findings in the case of a man aged 47 with Cushing's syndrome associated with a carcinoma of the thymus. The patient had a niece who was a pseudo-hermaphrodite and who died in infancy, and a nephew who had pubertas praecox and died in childhood. The patient himself died 8 hours after surgical removal of the thymic tumour, the blood pressure never rising above 58 mm. Hg. The cells of the tumour were epithelial-like. The suprarenal glands together weighed 28 g. and contained numerous small extracapsular adenomata 1 to 2 mm. in diameter; the anterior lobe of the pituitary gland contained typical hyaline basophil cells. After a review of the literature the author concludes that in Cushing's syndrome there is a tendency to carcinogenesis.

A. C. Crooke.

Correction.—Dr. Cumings writes that "on page 346 of the November issue under 'normal controls,' the third line of this paragraph reads '. . . blood creatinine was 0.5 mg./100 ml. or below. . . .' The word 'creatinine' should have been 'creatine.'"

J. clin. Path. (1951), **4**, 129.

WHITHER CLINICAL PATHOLOGY?

TRENDS AND OPPORTUNITIES*

BY

SIR LIONEL WHITBY

President of the Association of Clinical Pathologists, 1951–2

At the turn of the half-century, a little over a year ago, I was asked to contribute an article to the *British Medical Journal* on "Fifty Years of Clinical Pathology" as one in a series of articles on the same theme in other branches of medicine. The composition of that article was intensely interesting and highly self-educative. The study of the historical facts revealed certain milestones which themselves had given rise to trends in the subject which is our own. I have thought, therefore, that it might be of interest to try to think out what are likely to be the trends of clinical pathology in the years ahead, atomic bombs permitting, bearing in mind, first, the speed and trends of scientific advance, secondly, the impact of nationalization upon the practice of medicine, and, thirdly, what we can do to prevent the personal side of our particular craft from being submerged in the impersonality of the State.

Let us, in the first place, consider the speed of scientific advance in relation to what may be called the growing points of medicine. None can doubt that chemistry has come to be the foundation of nearly all scientific procedures, for it forms the fundamental basis of many of the laboratory manœuvres which hitherto, or until recently, we have performed by rule of thumb.

A few examples will make this concept abundantly clear.

For many decades we have used this or that medium for the cultivation of particular bacteria. These media were devised on an empirical basis—by trial and error, indeed, on much the same principles as governed therapeutics until relatively recent times. Now we appreciate that a particular medium will grow a particular organism because of the metabolic requirements of that organism, to wit, on account of the chemistry of its vital processes. Our empirical media contain this or that specific substance which is essential for the organism.

Likewise with histological staining. Whereas at one time a process of trial and error determined the best stain for a particular tissue and stains which provided the best specific contrast for this or that material, we now know that the virtues of these different stains depend upon their affinity for particular chemical groupings in the tissue itself. Cytochemistry or histochemistry is now a science of its own, and the application of a stain has as its object the demonstration of a specific chemical substance, rather than the production of a pleasant contrast histological picture.

* The Presidential Address given at the 45th scientific meeting of the Association of Clinical Pathologists in London in January, 1951.

I have in mind such procedures as the specific stains for ribose nucleic acid and other essential substances concerned with the metabolic processes of vital cells. Such stains, for example the Feulgen-reaction, enter also into haematology, and all will be familiar with the differences between vital and post-mortem staining.

Chemistry has indeed come to loom large in our workaday lives, and, since the basis of all physiological function as well as pathological dysfunction must be chemical change, it can be anticipated that the chemical pathologist will play a greater and greater part in the future in the diagnosis, control, and cure of somatic disease. I need hardly remark how important are the accurate estimations of the chemical pathologist in carbohydrate metabolism, renal function, liver function, gastric function, fat metabolism and fat balance, basal metabolism, and the various enzymatic changes that have been found to be associated with malignant or other changes in particular organs as well as with the complex problems of coagulation of the blood which involve numerous chemical procedures.

Let this then be the prime answer to the question posed in the title of my address, that the signpost for clinical pathology in the future is clearly pointing to the chemical aspect of the subject for those who wish to keep their individuality and to provide the personal opinion which has been the main aim of the clinical pathologist of the past. Nationalization, by creating regional bacteriological laboratories, may take from the clinical pathologist much, if not all, of the bacteriological work which he has performed in the past, and certainly when such work involves an epidemiological problem. Morbid histology is to a large extent an impersonal aspect of the subject. One is left with the certainty that the personal opinion of the clinical pathologist will always be needed in the fields of haematology and biochemistry. Pathology in these fields cannot be divorced from a clinical opinion, and it is upon these aspects of medicine that the budding clinical pathologist should concentrate. But if this be the prime answer to the question I have posed, what else can we do to save our souls from routine, to exercise our intelligence in relation to the numerous opportunities which are presented to us, almost daily though perhaps unnoticed, and to advance our subject so that it comes to occupy the forefront of medicine, as indeed it should, for our branch of medicine is in the British tradition, the natural gradual evolution which combines the clinical acumen of the past with the somewhat mechanical science of the present. I make no criticism of my numerous American friends when I say that many of them have tended to over-emphasize the latter, whereas many British physicians still insist upon living in the days of Sydenham and Heberden.

We have, I am sure, unrivalled opportunities for advancing the science and art of medicine, so long as we are ever alive to the significance of what we have to study and so long as we regard our work as having this potential bearing. To show what I mean, I propose to devote some time to making a number of provocative pronouncements about the nature of leukaemia—a disease in which I have been interested for a quarter of a century, a disease with whose tragedies we are all too familiar. I make these observations because it is the function of a presidential address to stimulate thought and to show what problems can be presented, even though they may not be solved, by constant pondering on the material which passes through one's hands almost daily. I do not anticipate that I shall tell you anything new, but I may be able to present to you, from another angle, the facts which you already know.

In this dread disease, leukaemia, there are numerous manifestations which are recorded as isolated facts in textbooks of medicine without any emphasis upon the conclusions which can be drawn from them and without any attempt to bring them into a perspective where they can be used to discuss the nature of the disease. The spectacular and absorbingly interesting haematological changes have received the most minute attention, and this alone has tended to overshadow the morbid anatomy and basic pathology and also to divert attention from certain clinical facts which may provide the clue to the elucidation of the cause and the devising of appropriate treatment. The development of modern methods for studying cell growth and cell metabolism—to wit, the vital chemistry of the living cell, the aspect of clinical pathology which I have emphasized as being the signpost for the future—has also thrown further light on the problem of leukaemia by revealing the nature and potentialities of the leukaemic cell which have usually been regarded as undoubtedly malignant on account of the invariable fatality of the disease and, in the clinical field, the obvious superficial resemblance to cancerous conditions.

First let me take up some points on the clinical side. All leukaemias are characterized by an abnormal proliferation of leucopoietic tissue in the bone-marrow, the proliferation being at an immature level which usually, but not always, results in the appearance of large numbers of leucocytes in the peripheral blood. Many of these are immature, and the degree of immaturity bears a rough relationship to clinical severity. The most acute types are characterized by a preponderating, if not an exclusive, presence in the blood of cells so primitive that they often defy exact differentiation. Likewise with the terminal phases of the chronic types which are often acute from both the clinical and the haematological aspects. These well-known facts should give food for thought.

In malignant disease in general we recognize acute fulminating growths such as the mesoblastic sarcomata, and at the other end of the scale the chronic, long-standing scirrhous types of carcinoma. This then is common ground with leukaemia. But it is difficult to think of examples of orthodox malignant disease in which the chronic scirrhous type suddenly and terminally takes on a fulminating character. We know also that leukaemia may remit spontaneously, or from the intrusion of other complications, or as the result of treatment. But in other malignant disease spontaneous remission is so rare that when it occurs it raises grave doubts as to the accuracy of the original diagnosis. Thus, there are certain clinical features of leukaemia which seem to separate it from orthodox malignant disease. However, there are certain features common to both, for example, the progressive anaemia, the associated cachexia, and the invariable fatality.

Let us now take up a haematological point. We are thoroughly familiar with two types of the disease which we designate as leukaemic or aleukaemic, according to the state of the peripheral blood, but these do not differ greatly in clinical features save that in aleukaemic cases the lymph nodes, the spleen, and the liver may not be so grossly enlarged at the outset. This fact, that the disease may be leukaemic or aleukaemic, is accepted as a commonplace, a mere variation in manifestations. It has not given rise to argument and serious discussion. Yet the fact that two types exist suggests the derangement of two different processes which, on rational grounds, should be physiologically interlocked. I refer, of course, first to the process of maturation and, secondly, to the process of release into the circulation. We are

woefully ignorant of the nature of any materials which we could confidently call specific maturation factors, factors, say, which cause the myeloblast to mature to the granulocyte, and we know even less of the forces or factors which regulate the release of granulocytes into the circulation or which restrain the myeloblast from so doing. Thus, in frank leukaemia, there appears to be inhibition or retardation of maturation in whole or in part, combined with lack of control over release, since vast numbers of cells, including primitive ones, flood the circulation.

In the aleukaemic disease, on the other hand, lack of maturation is a prominent feature, but the release mechanism is largely intact in that the numerous primitive cells in the marrow may not reach the circulation except in small numbers.

These facts make it abundantly clear that we know little or nothing of the physiology of leucopoiesis, and I am convinced that we shall not understand leukaemia until some of these basic physiological facts have been determined. If half the time and energy which have been devoted to devising and exploiting empirical remedies for the symptomatic treatment of the disease, reducing the leucocyte count, had been expended on basic physiology and pathology, we might well be much nearer to rational treatment.

I may mention in passing that sternal puncture has revealed how much more common is the disease than was at one time supposed. There has been a parallel decline in the number of cases diagnosed as aplastic anaemia which, in a primary form, must now be considered to be extremely rare. Many cases of aleukaemic leukaemia were previously misdiagnosed as aplastic anaemia. Even so, this is not sufficient to account for the remarkable increase in the incidence of leukaemia during the past 25 years, as shown by published statistical studies as well as the experience of most of us in practice. Even more remarkable has been the increase in the monocytic types, especially the Naegeli form. No one has yet suggested an explanation.

Let me now comment on one or two points of factual pathology and morbid anatomy which again are well known, but which are accepted as dull and ordinary and so have not given rise to the discussion which they merit.

With the fully developed leukaemic process all tissues of the body may be infiltrated with leucocytes, and in some sites these may undergo proliferation. It is this feature which gives rise to the protean clinical manifestations of the disease, so that a leukaemic patient may present at almost any of the special departments of a large hospital. I need hardly elaborate this point. Sufficient to say that the leukaemic infiltration may be gross, giving rise to pressure or derangement symptoms, or may be moderate or may be so slight, in some aleukaemic states, that even pathologists argue about the diagnosis. I would remind you too that the leukaemic cells have a characteristic distribution in the liver according to the type of the disease. We recognize the portal distribution in lymphatic leukaemia, the intercellular and intrasinusoidal distribution in the myeloid type, and the subcapsular collections in the monocytic form. In no other malignant disease in which the malignant cells are distributed by the blood stream does this peculiarly constant deposition occur.

Furthermore, as an additional argument against the malignant nature of the cells themselves, one must acknowledge that the infiltrating cells do not multiply freely, nor do they invade and destroy surrounding tissue, nor do they give rise to

a stromal reaction. The leukaemic cells may multiply in certain sites, but the nature of the multiplication suggests a comfortable symbiosis, rather than a malignant invasion, and the sites in which this multiplication most freely occurs are in the organs in which foetal blood production occurs, the spleen, the liver, and the kidney, organs in which sinusoidal spaces are abundant.

Yet, on the other hand, the rule about lack of invasive and destructive qualities is not absolute. The rare chloromatous tumour invades and destroys whilst the lymphosarcomatous tumour, the cells of which are histologically indistinguishable from the cells of lymphatic leukaemia, and which itself sometimes terminates with the blood picture of lymphatic leukaemia, both destroys tissue and gives rise to invasive and destructive metastases.

So far, I have dealt with nothing abstruse or experimental, but have merely commented upon known facts which we who see both sides of the case, the clinical and the pathological, have ample opportunity to observe, ponder upon, and correlate.

I want now to mention one or two experimental points which have arisen from these thoughts and which have been carried out by a young research student working in my department in the fields of tissue culture and cytochemistry, the results of which were communicated by him to the last International Congress of Haematology.[1]

It has long been known that leukaemic cells develop into normal cells when cultured *in vitro*, but a further interesting fact is that they proliferate at the same rate as normal cells. Furthermore, tissue culture results show quite readily that primitive cells which in the *in vivo* conditions within the body of a leukaemic patient are unable to mature, nevertheless do so quite readily *in vitro*. And, finally, when such leukaemic cells are examined *in vitro* they present several points of interest. Myeloblasts may mature into granulocytes. On the other hand, if the culture be contaminated with some innocuous bacterium the cells differentiate into macrophages which ingest the bacteria. No one can quote me an example of an orthodox malignant cell which retains its capacity for differentiating into a highly specialized type of non-malignant cell given the appropriate stimulus.

This kind of result suggests that the fundamental defect in leukaemia does not lie with the cells themselves which, given an altered environment, display all the properties of normal cells in respect of maturation, rate of proliferation and, above all, a capacity to differentiate in response to appropriate stimuli. What then can we learn about the leukaemic environment? Here again we can obtain hints from the experimental field, from clinical observations, and from straightforward post-mortem examination. In the experimental field one can observe the effect of normal cells when implanted into a leukaemic subject. We are all familiar with the leukaemic nodule in the skin which is sometimes a prominent feature in an individual case. A leukaemic nodule can readily be produced in the skin of a leukaemic patient by the subcutaneous injection of a small quantity of normal marrow; the normal cells proliferate and undergo mitosis. If, however, leukaemic marrow be implanted beneath the skin of a non-leukaemic person no nodule is formed.[1] From this one can deduce that normal cells placed in a leukaemic environment behave like leukaemic cells, whilst the reverse is also true.

[1] Hoogstraten, J. (1950). Proceedings of the Third International Congress of Haematology. Grune and Stratton, New York.

Turning now to the clinical field, we know that leukaemic blood has several times been transfused in considerable volume, either by accident, or by design, in cases of agranulocytosis. But in no case has the disease become implanted in the recipient, whilst the transfused leukaemic cells have behaved as normal cells. Yet how easily do orthodox malignant cells become implanted in the site of an excision. From the clinical field also I think of what is well known to those of us who see many cases of leukaemia, namely, the intrusion of an acute infective stimulus, such as a carbuncle, which may cause a rapid and dramatic remission in a case of myeloid leukaemia. The sudden application of a dominating stimulus, an infection, may immediately cause the marrow to revert to normal. This never occurs in malignant disease. Likewise, with spontaneous remissions which all must acknowledge as a clinical fact. What has happened in such cases? It must be that the environment has altered, that whatever metabolic fault or deficiency is responsible for the disease, has shown a flicker of return to normality.

We should also take note of the clinico-pathological fact that when leukaemia is manifested it appears to arise in all places at once. So far as I know, there has been no record in all the hundreds of thousands of necropsies which have been performed of the finding of a local leukaemic marrow lesion suggestive of the inception of the disease. When the disease occurs the sinister hyperplasia is orderly, uniform, and progresses along the lines of physiological expansion as with infection, though at a more primitive level.

Ordinary malignant disease, on the other hand, begins as a local tumour which afterwards disseminates. It is, indeed, a striking fact that in leukaemia there is no question of local growth. Whatever bone is examined shows that the leukaemic process arises simultaneously in all, and this is a pathological fact which is surely unique so far as malignant conditions are concerned. It is highly suggestive that leukaemia arises from a somatic or metabolic environmental fault.

Now let me turn to the treatment of leukaemia and make certain deductions, criticisms, and suggestions from this aspect of the disease. In the first place, I have already drawn your attention to the fundamental difference between aleukaemic leukaemia, when the mechanism of maturation is disturbed but not the release mechanism, and frank leukaemia when both processes are involved. When the release mechanism is intact we are often unable to use some of our empirical remedies, such as *x* rays, urethane, nitrogen mustards, or the pterins. But when the release mechanism is disturbed in frank leukaemia then we get a form of the disease which is very amenable to anti-mitotic empirical remedies. There is therefore more than an academic difference between leukaemic and aleukaemic leukaemia. Let me next remind you of a modern rediscovered fact, namely, the, alas, only temporary effect of a massive transfusion of normal blood to the acute leukaemic case, which, *per se*, has generally been regarded as rampantly fatal. The same results, in my own experience, can be achieved with a massive transfusion of fresh plasma, though not with the reconstituted dried product. Is there therefore some inhibiting factor in the blood of all of us which prevents the development of leukaemia or does leukaemic blood lack some factor without which this dread disease develops? Is the disease therefore no more than an exhausting impotent hyperplasia, rather than a true malignant process? If this last be true, then the principles of modern treatment, which are designed to inhibit mitosis rather than remedy a deficiency,

must be fundamentally wrong. Here also in this temporary response to transfusion is another divergence from orthodox malignant disease, for cancerous processes do not remit after simple transfusion. Here too is the germ of hope for the future. For if this relatively natural and exceedingly conservative form of treatment can induce a remission, what is it that is contained in normal blood which brings about this sudden change—a change so remarkable that even the marrow may revert almost to normal. Could this factor in normal blood be positively identified we should be on the threshold, if not within the door, of a method of treatment far more realistic and rational than antimitotic measures such as assaults with such substances as variants of mustard gas or *x* rays which, indeed, seem to be mainly aimed at a symptom rather than at a cause.

Indeed, with new and empirical remedies we should always bear in mind that the mere reduction of a leucocyte count is no more than symptomatic treatment which may neither induce a clinical remission nor really get at the fundamental cause of the disease. We do not put out a fire by devising methods for abolishing the smoke; we do not cure an infection by abolishing the associated fever, and we shall not cure leukaemia by abolishing mitosis. The problem is really to find the something which controls and regulates mitosis, and this returns the matter to the field of chemistry which I have suggested is the main route which our subject will travel in the years to come. To my mind, the significant pointers in the field of treatment are the action, though far from satisfactory, of the pterins, and of blood transfusion; the newer knowledge that leukaemic cells contain only 10% of the normal amount of zinc and the effect, in some cases, of adrenocorticotrophic hormone. These seem to me to offer more hope of extension and exploitation than the building of bigger and more powerful death-dealing machines of highly penetrating types, since they provide a more physiological approach and even give a hint that leukaemia may eventually be categorized among the deficiency diseases in company with pernicious anaemia and diabetes.

I will admit that I am open to criticism for departing a long way from the title of my address and for having virtually stolen an opportunity to talk almost exclusively on one single subject. I have done so deliberately, for the main substance of my address is given as an example of the opportunities that come the way of the clinical pathologist. Many of you could have given other examples in relation to the diseases in which you have had a lifelong interest, and indeed I myself might have chosen several other examples and dealt with them in less detail.

Whither clinical pathology? I can say quite definitely that the subject will go in the direction in which we make it go, and the opportunities for advancing the subject of medicine are more ready to the hand of the clinical pathologist than to any other section of the profession.

I commend these thoughts to you for your earnest consideration. Those of us who are elderly would be well advised to interest ourselves in the elements of organic chemistry which we have long ago forgotten. Those of us who are still in their prime should have no difficulty in addressing themselves to this task. Those of us who are young and those who are about to enter the field of practice as clinical pathologists cannot avoid the issue. The one essential in clinical pathology is to be one jump ahead of the times, and to me it seems obvious that the future

of successful diagnosis and therapeutics lies in the field of biochemistry with biochemical control of such therapeutic agents as are found to have a specific effect in individual diseases. This is already the case with pernicious anaemia, diabetes, and other metabolic diseases. I anticipate that it will be the case with diseases amenable to A.C.T.H., and whatever materials of like nature may be discovered, and, who knows, even with leukaemia or with cancer and comparable processes which are nowadays considered to be malignant.

The future of our subject lies with ourselves, and we must never allow it to degenerate to the mechanical level of a technique. Our success in the past has been due to the individual and personal factor, to our ability to give a clinical as well as a pathological opinion. Clinical work is essentially personal. The pathological aspect must be the same. This indeed is one of the danger points arising from nationalization. For though there are still many people who will pay a private fee for a single clinical opinion, there are none, or almost none, who will pay for the host of pathological and other examinations which are ordered as the result of such a consultation. Our laboratories are therefore in danger of being overwhelmed with routine work, much of it unintelligently demanded, and with such an overburden the personal element disappears, to the detriment of original work, proper thought, and also status. It is vital that this should not happen if the craft of clinical pathology is to survive or is to make its contributions to medicine or to maintain the status which it has laboriously built up in the past 25 years. The only remedy is an increase in the establishment of fully qualified graduate staff, so that there are sufficient for each and every specimen to be the personal responsibility of the pathologist and there is sufficient time for a man to give thought to his problems. In my time I have been guilty of signing many reports on specimens which I have never seen or never questioned. I have been fortunate in having a reliable technical staff who would always refer a doubtful report. Nevertheless, I have occasionally endured the humility of having missed a difficult haematological diagnosis from lack of personal supervision, and I am sure that it is this kind of danger which nationalization threatens. In brief, it is of little use for us to clamour for specialist status with merit award if we do not personally justify that status and the award both in our everyday work and in our contributions to the advance of knowledge derived from the material which we handle.

Let us not leave our work to technicians and registrars, let us keep the personal element alive, the clinical opinion and the intelligent application of laboratory procedures to the understanding of the individual case ; to wit, a modern physician. If this is done the Association will have strong and natural links with its legitimate parent—the Royal College of Physicians—and the subject will go from strength to strength in the fabric of the now irreversible nationalized service.

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POLYARTERITIS NODOSA AND THE "RHEUMATIC" DISEASES

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Polyarteritis nodosa has been the subject of comprehensive reviews covering most of its clinical and histological aspects (Smith and Zeek, 1947; Miller and Daley, 1946; Davson, Ball, and Platt, 1948). There are, however, four points which require clarification, and they are discussed in the present paper: (1) the early changes and the question of the so-called "fibrinoid" substance; (2) some histological criteria reputed to distinguish polyarteritis nodosa from the rheumatic and "pararheumatic" diseases, including "temporal arteritis" ("pararheumatic" is used in the sense defined by Teilum, 1946); (3) polyarteritis nodosa as the basis of "Löffler's syndrome" (eosinophil-celled alveolitis) associated with dermatoses; (4) the factor of bacterial hypersensitivity in the causation of polyarteritis nodosa.

The material consists of 18 cases, 13 men and five women. Their ages ranged from 38 to 69, one-third of the subjects being between 46 and 48 years.

Eight biopsies were taken: four from muscle, two from skin nodules, one from sympathetic ganglion, and one from the temporal artery. Ten necropsies were performed.

The following are the preferential sites in which polyarteritis lesions were found post mortem.

Kidney	6
Myocardium	2
Tongue	4
Gut and gall bladder (1 case ?)	2
Brain	2
Lung	2
Periadrenal fat tissue	6
Probable connexion with sulphonamides	3

The Early Changes

It is generally agreed that the basic change in polyarteritis nodosa is the increased permeability of the vascular wall. If this is mild, fluid penetrates the intact endothelium and causes subendothelial oedema. In more severe cases fibrinogen also escapes and the subendothelial material stains as for fibrin. In the still more severe case, there is actual necrosis of the vascular wall. A patch of fibrin may lie in the necrotic vascular wall, extending into the surrounding tissues, or give the appearance of a solid plug of fibrin filling the breach (Fig. 1) P. As a rule, the subendothelial swelling is patchy or eccentric, but in severe cases it affects the entire circumference (Fig. 2) F. An early fragmentation and later disappearance of parts of the internal elastic lamella is characteristic (Fig. 2*a*),

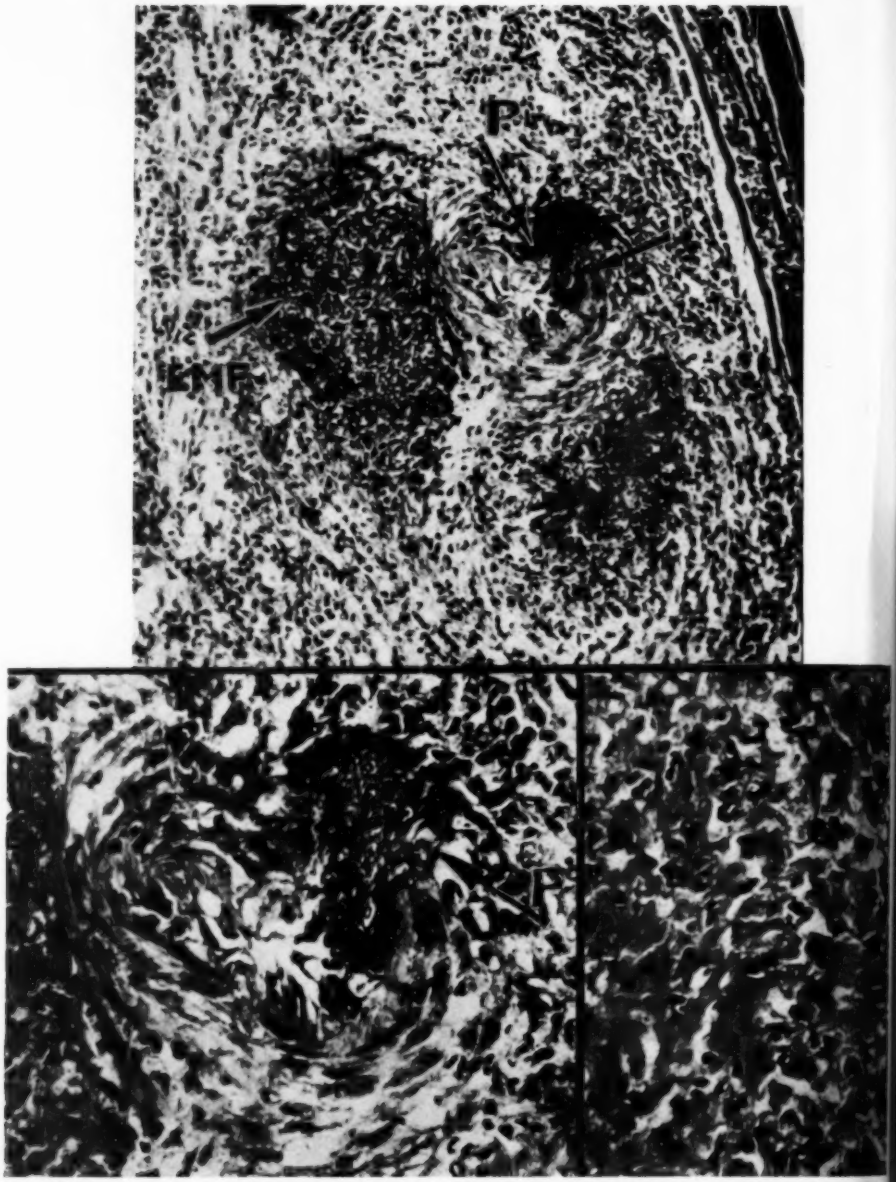


FIG. 1.—Section of tongue of male aged 47 years (P.M. 50/66) with polyarteritis nodosa. Haematoxylin and eosin. Breach in arteriolar wall plugged by fibrin (P). Two extra-mural foci of conglutinated collagen (EMF) × 120. Inset left (P) × 250. Inset right (EMF) × 250.

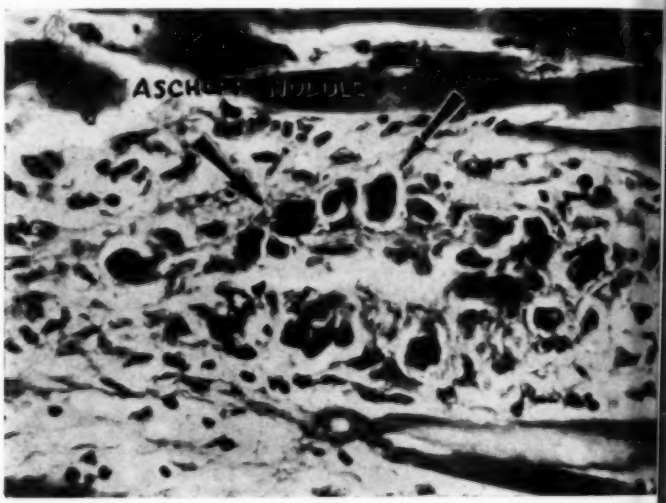
FIG. 2.—Section from same subject as in Fig. 1 stained with phosphotungstic acid-haematoxylin. Fibrinoid necrosis and fibrin deposition along the vascular circumference. Fibrinoid replacing original vascular wall (F). Fibrin deposited inside vascular wall (I.F.). Fibrin deposited outside vascular wall (E.F.) × 100.

FIG. 2

FIG. 2*a*.—Section from same subject as in Figs. 1 and 2 stained with Weigert's elastica stain. Peripheral muscle. "Hair-pin" artery. Gap in the internal elastic lamella caused by fresh fibrin necrosis × 120.

FIG. 2*a*

FIG. 13.—Section from Case 1 stained with haematoxylin and eosin. Myocardium (top) eccentric fibrin infiltration in vascular wall (F). Aschoff body (bottom) developing around a vascular lumen showing small patches of fibrin and large cells with fragmented nuclei (× 350).



and the finding of such gaps is helpful in the diagnosis of polyarteritis nodosa scars (Bauke and Kalbfleisch, 1934 ; Ball and Davson, 1949 ; see also page 149).

The degenerative changes in the vascular wall are usually followed by the perivascular collection of inflammatory cells. Among the latter, eosinophil cells may be predominant, but they are not invariably present during the destructive phases of the disease and, as shown below, may occur at a late stage when the acute changes are burnt out.

Deposition of fibrin is conspicuous in the early stages and, to a lesser degree, in the later changes in polyarteritis nodosa, but, as Fishberg (1923) noted, the material deposited in the vascular wall does not entirely consist of fibrin. A

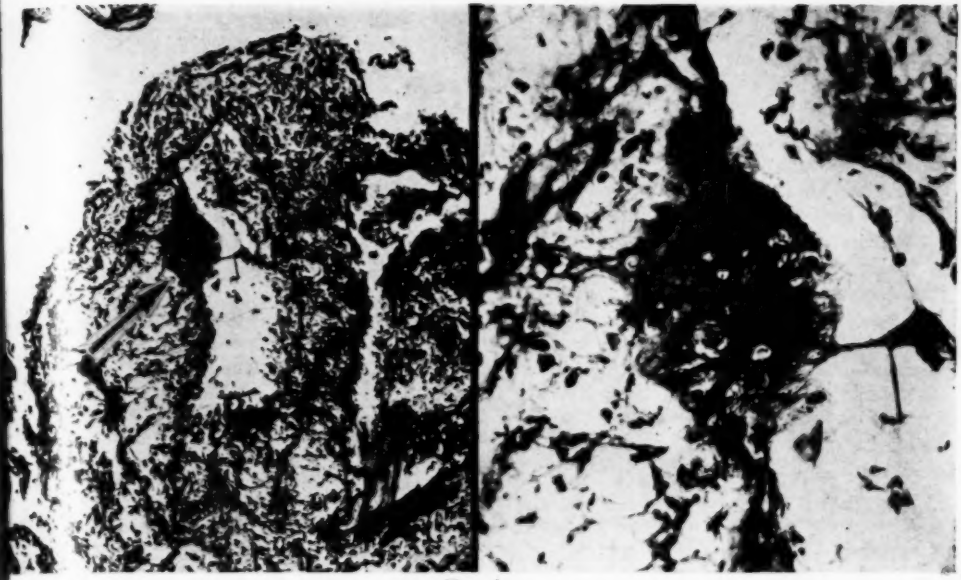


FIG. 4

FIG. 4.—Section from Case 2 stained with haematoxylin and eosin. Subcutaneous nodule. Patch of fibrin bordering a vessel (× 85 and × 300).

FIG. 5.—Section from Case 2 showing patches of fibrin scattered in connective tissue. Most of the cells are eosinophils. Top: × 85. Bottom: × 250. Haematoxylin and eosin.

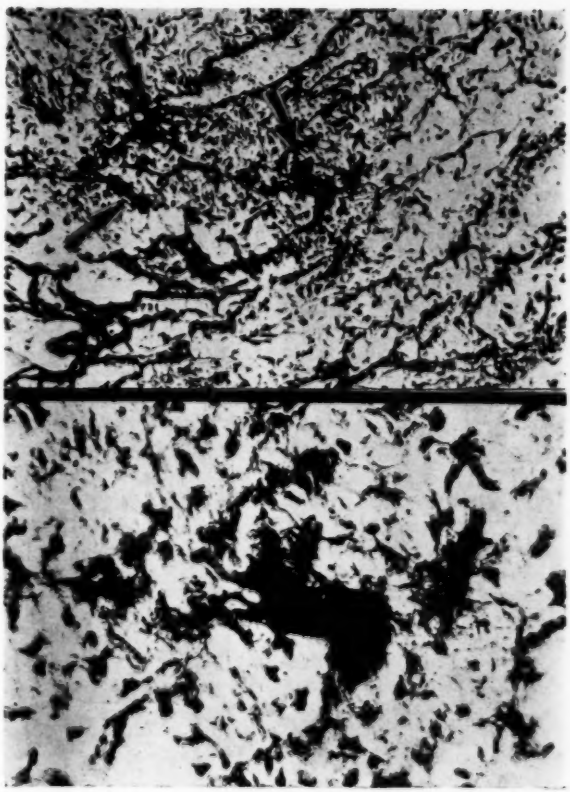


FIG. 5

characteristic finding in the material here presented was the deposition of fibrin rings inside as well as outside the "fibrinoid" necrotic part of the vascular wall (Fig. 2) EF and IF. The latter shows gaps through which the internal and external fibrin deposits appear to communicate. The fibrin-negative central part (Fig. 2) F seems to be quite different in structure from the frank fibrin that is deposited inside and outside it. On the other hand, in the polyarteritic lesion fibrin-negative material is found more commonly at the periphery than in the centre of the deposit. This ill-defined homogeneous substance remains unstained by Weigert's method for fibrin, phosphotungstic-acid haematoxylin, and the acid picro-Mallory method recommended by Lendrum (1949). All this seems to favour retaining a special term to describe it. As it has neither the staining reactions nor the morphology of fibrin, the use of the term "fibrinoid" may raise objections. "Hyaline" may be preferable in view of its homogeneous "glass-like" appearance.

The subendothelial deposition of "hyaline" is not in itself indicative of early polyarteritis nodosa. It has been described as a change preceding "arteriolar and capillary platelet thrombosis" (Gore, 1950). On the other hand it can be secondary to capillary thrombosis and form a certain stage in the organization of a fibrin thrombus. This has been shown in "acronecrosis" following subacute bacterial endocarditis and in platelet thrombosis (Pagel, 1949). There is no evidence of any primary thrombosis in early polyarteritis nodosa, and, moreover, subendothelial hyaline deposition, without thrombosis, can also be observed in various other conditions, for example in dermatomyositis, fulminant tuberculous septicaemia, and in the response to intradermal injection of heat-killed streptococci in patients with rheumatic fever (Pagel, 1949; Pagel, Woolf, and Asher, 1949; Humphrey and Pagel, 1949).

The Polyarteritis Nodosa Changes in Rheumatic and "Pararheumatic" Conditions

The histological changes in polyarteritis nodosa are reputed to be typical of this disease and to be distinguishable from the lesions of rheumatic and "pararheumatic" conditions (lupus erythematosus disseminatus, dermatomyositis, scleroderma, "arteritis granulomatosa allergica," temporal arteritis, etc.). It is probably true to say that the basic changes in all these diseases are closely similar, if not identical, although there are differences in the frequency and distribution of the lesions in the different diseases.

Fibrinous Infiltration of the Vascular Wall.—This change, which is virtually a *sine qua non* of the early phase of polyarteritis nodosa, is in fact a common finding in "rheumatic arteritis." It has even been said that "the picture of vascular damage in rheumatic disease recalls polyarteritis nodosa so clearly that one has to ask if this is not the same disease or, at least, their morbid anatomy leads to the same result" (Klinge and Vaubel, 1931; VonGlahn and Pappenheimer, 1926). Two examples of this change have been seen.

Case 1.—A boy, aged 16, had had recurrent rheumatic myocarditis for four and a half years, with mitral and aortic stenosis. Repeated blood cultures were sterile. At necropsy (P.M. 1945/2) a large, elongated heart was seen. There were many partly calcified vegetations on the mitral valve, and aortic stenosis due to scarring of the valves. The lungs were rubbery, showing histological changes typical of so-called

"rheumatic lung." Histological examination of the myocardium showed (*a*) patches of fibrin and fibrinoid matter in the interstitial tissue and in the intima of arterioles (Fig. 3 F), with or without peripheral cell collection ("early rheumatic infiltration"); (*b*) multiple "Aschoff-bodies" of the spindle as well as of the coronal type; some of these developed around patches of fibrin in the vascular wall (Fig. 3).

Case 2.—A boy, aged 14 years, had had chorea and rheumatic fever seven years previously. He was admitted with a history of 14 days' pain in both shoulders and knees. He had atactic speech and occasional facial twitching. He had had a few subcutaneous nodules on the right olecranon for some time. Six days after admission a fresh crop of nodules developed on the hands, wrists, and around the ankles. One of these (biopsy S.D. 1602) showed patches of fibrinoid matter, with cores of fibrin, scattered in the connective tissue in typical rheumatic fashion (Fig. 5). But the vascular changes (Fig. 4), which showed in addition widespread infiltration with eosinophils, were characteristically those of polyarteritis nodosa. Clinically, however, the diagnosis seemed to be clearly that of acute "rheumatic fever."

In the "pararheumatic conditions"—lupus erythematosus disseminatus (see especially Figs. 8–10 in the paper by Klemperer, Pollack, and Baehr, 1941) and dermatomyositis (Fahr, 1921; Pagel *et al.*, 1949)—vascular changes occur which are indistinguishable from those of polyarteritis nodosa.

"Collagen Disease."—This is a degenerative and exudative change affecting the collagen fibres, which become waterlogged, conglutinate, and lose their normal staining affinities. There is also exudation of fibrin in the collagen tissue.

This alteration of collagen is found in lupus erythematosus disseminatus (Klemperer *et al.*, 1941) and in dermatomyositis (Pagel *et al.*, 1949). It is also present in the "early rheumatic infiltration" in the two cases described above. Indeed, there does not seem to be any *structural* difference between "early rheumatic infiltration" (Klinge, 1933) and this so-called "collagen disease." It is not mere coincidence that when the relationship of these collagen changes to the argyrophil reticulin is studied, it is found to be identical in rheumatic disease (Klinge, 1933), polyarteritis nodosa, and the "pararheumatic" diseases. In all there is a *peculiar resistance of the argyrophil reticulin* even in the middle of a zone of gross change, as is shown in Fig. 6. This section is from the tongue of a male aged 47 with classical polyarteritis nodosa terminating in cerebral haemorrhage (P.M. 1950/66), and shows a typical patch of vascular fibrin-necrosis in which the silver impregnated reticulin fibres remain intact. The abnormality of the collagen fibres of the vascular wall is clearly shown in other sections by their peculiar staining reactions with Van Gieson's and Mallory's methods.

A further point of similarity between polyarteritis nodosa and the rheumatic and "pararheumatic" conditions is that paravascular foci occur in all. An example is given in Fig. 1 from a case of polyarteritis nodosa. In all these varied diseases the paravascular lesion also shows an intact reticulin, and thus conforms to the early rheumatic lesion (*Frühinfiltrat* of Klinge or "reticular Aschoff body" of Gross and Ehrlich, 1934). I have confirmed this similarity in polyarteritis nodosa and in dermatomyositis.

Occasionally, in polyarteritis nodosa, the *paravascular* lesion, especially if cutaneous, goes on to necrosis. The area of necrosis is full of chromatin debris

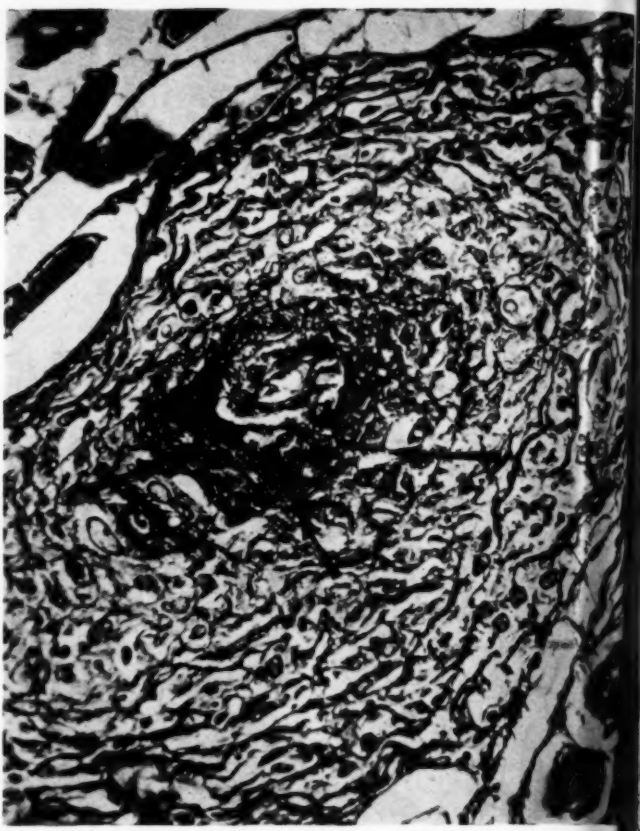


FIG. 6.—Section of tongue of the same subject as in Fig. 1 showing a trellis of preserved reticulin fibres in an area of perivascular fibrin necrosis. Wilder preparation (× 200).

and is surrounded by epithelioid cells and Langhans giant cells, and thus resembles a tuberculous lesion (Fig. 7). No acid-fast bacilli were found. Similar tuberculoid skin nodules have been mentioned in a case with transient infiltrations of the lung with pleural effusion described by Livingstone (1943–4). Structurally they seem to be identical with focal lesions observed in the upper respiratory tract (Howells and Friedmann, 1950), in the lung (Banowitch, Polayes, and Charet, 1942; Baggenstoss, Bayley, and Lindberg, 1946; Bergstrand, 1946; Smith, 1948), in the heart muscle (Rose, Littmann, and Houghton, 1950), and in the liver (Zuelzer and Apt, 1949), in cases with a variety of clinical symptoms but all showing excessive eosinophilia. It is therefore perhaps significant that the following case with tuberculoid lesions had gross eosinophilia.

Case 3.—A woman, aged 49, with a history of bronchitis and asthma for 20 years, was admitted with indefinite gastric complaints, and on examination showed a leucocytosis (14,500–18,000) of which up to 63% of the white cells were eosinophils. Five weeks before admission she had had an irritating rash on the legs, extreme sweating at night, weakness of the hands and slight weakness of the rest of the body, numbness in the feet up to the knees, foot drop, complete loss of sensation for some time, and transient tender lumps, reaching the size of peas, in the legs and forehead. She showed

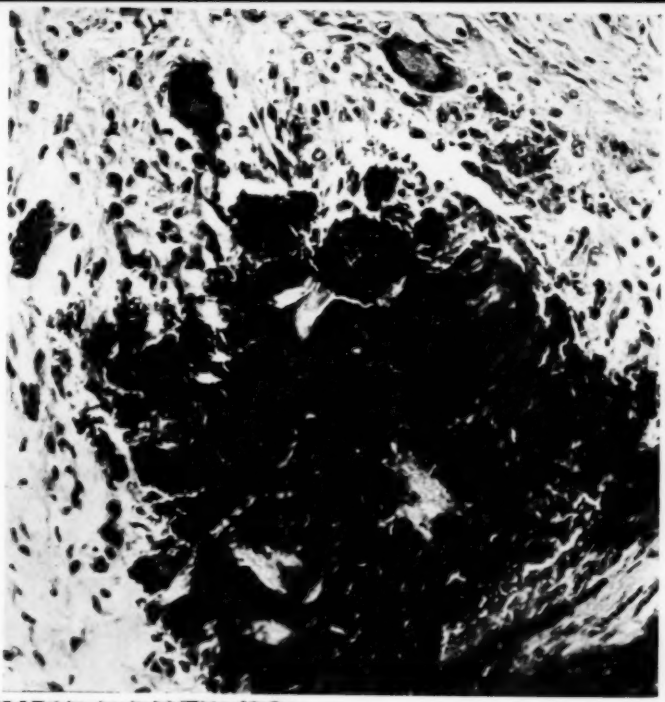


FIG. 7

FIG. 7.—Section of skin nodule of woman aged 49 years (S.D. 2564 and 2682) with polyarteritis nodosa, showing granuloma with central necrosis and giant cells of the Langhans type in the periphery. Haematoxylin and eosin (× 350).

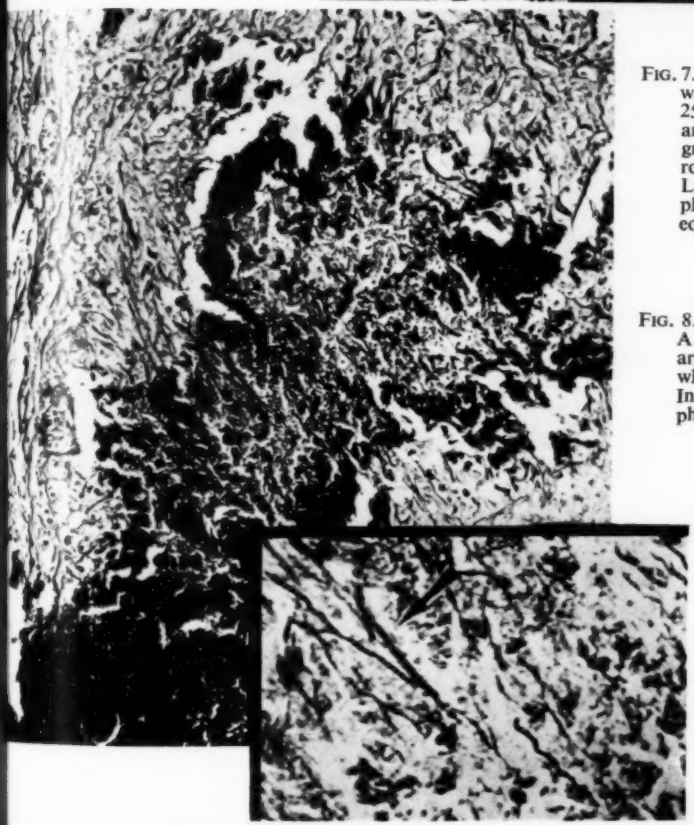


FIG. 8.—Same section as Fig. 7. A Wilder preparation for argentaffine reticulin fibres, which are preserved (× 100). Inset is high-power (× 200) photomicrograph.

FIG. 8

irregular pyrexia and a persistent eosinophilia, which gradually came down to 12%, with a total white count of 6,500 per c.mm. The results are tabulated.

Date	White Blood Cells	Eosinophils
13/9/43	18,000 per c.mm.	48% = 8,640 per c.mm.
16/9/43	14,500 ,, ,,	63% = 9,135 ,, ,,
27/10/43	13,500 ,, ,,	33% = 4,500 ,, ,,
1/2/44	10,500 ,, ,,	46% = 4,830 ,, ,,
7/3/44	19,000 ,, ,,	30% = 4,700 ,, ,,
24/3/44	6,000 ,, ,,	16% = 960 ,, ,,
16/5/44	7,000 ,, ,,	26% = 1,820 ,, ,,
16/7/44	6,500 ,, ,,	12% = 760 ,, ,,

Occult blood in the stools was strongly positive.

Polyarteritis nodosa was diagnosed, but it was noted that the fever and pulse were not typical and that the patient was not as ill as might be expected. Herpes zoster affecting the seventh and eighth dorsal segments appeared shortly after admission. Biopsy of skin from a foot (10/10/43) showed extensive perivascular, perifollicular, and periglandular infiltration with eosinophil leucocytes and a few plasma cells and mononuclear cells. The same changes were present in the subcutaneous adipose tissue.

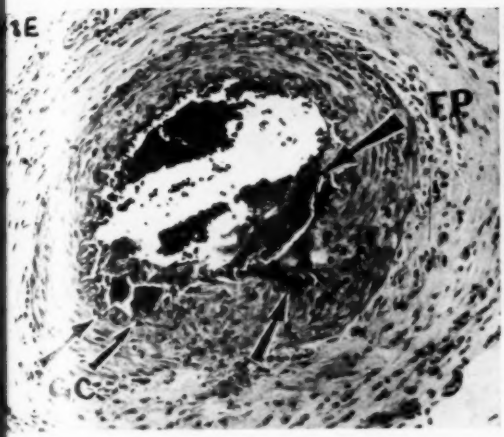
Biopsy from skin and deltoid muscle (23/10/43) showed no appreciable changes in the skin except subpapillary oedema with smoothing out of the corpus papillare. A few plasma cells were scattered in the layers of the cutis. There were no vascular or perivascular changes, or changes in muscular tissue. Biopsy from skin of a foot (1/4/44) showed that the dermis contained a well-defined nodule with central necrosis and a number of giant cells of the Langhans type in the periphery (Fig. 7). A ring of lymphocytes and thickened connective tissue surrounded the focus. Wilder preparations showed the reticulin fibres to be occasionally fragmented but preserved (Fig. 8), although collagen fibres seemed to have disappeared. Some arterioles in the periphery showed fibrinoid necrosis in the intima.

The patient improved considerably and, although she was still well when seen four years later, it is considered that this was, in fact, a case of polyarteritis nodosa.

Granuloma.—The initial vascular changes in polyarteritis nodosa, the fibrinoid necrosis in the subintimal layers, are soon followed by cellular aggregation, and in some cases go on to frank granuloma. One type of this is rather in the nature of an extension of the necrotic region described above (Fig. 7). The other type of tuberculoid granuloma in polyarteritis nodosa occurs in the affected vascular wall itself (Fig. 9).

Case 4.—A man, aged 47, had classical polyarteritis nodosa in all stages, and the sections were taken from a branch of the tibial artery. The granuloma with prominent giant cells (GC), both of the Langhans and foreign body type, had developed around a patch of fibrin (FP), adherent to the intima. This parietal fibrin-thrombus, together with the granuloma, filled a gap in the vascular structure which was outlined by a defect of the internal elastic lamella. Elastic debris acting as foreign bodies may possibly explain the granuloma and the presence of giant cells.* No such debris was found, however, in the present case.

* The connexion of giant cells with elastic debris is clearly seen in " temporal arteritis " (Harrison, 1948), and in the lung of pulmonary haemosiderosis (Lendrum, Scott, and Park, 1950).



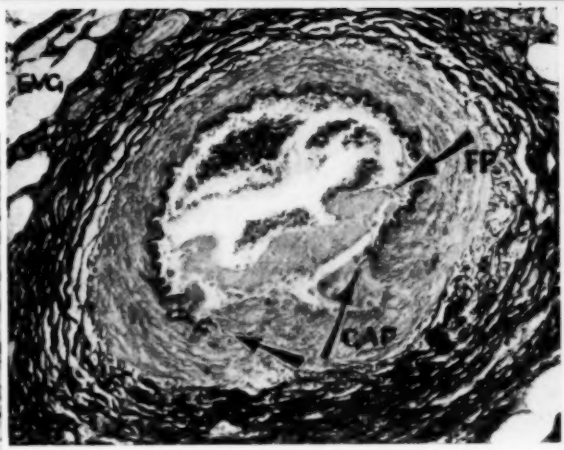


FIG. 9

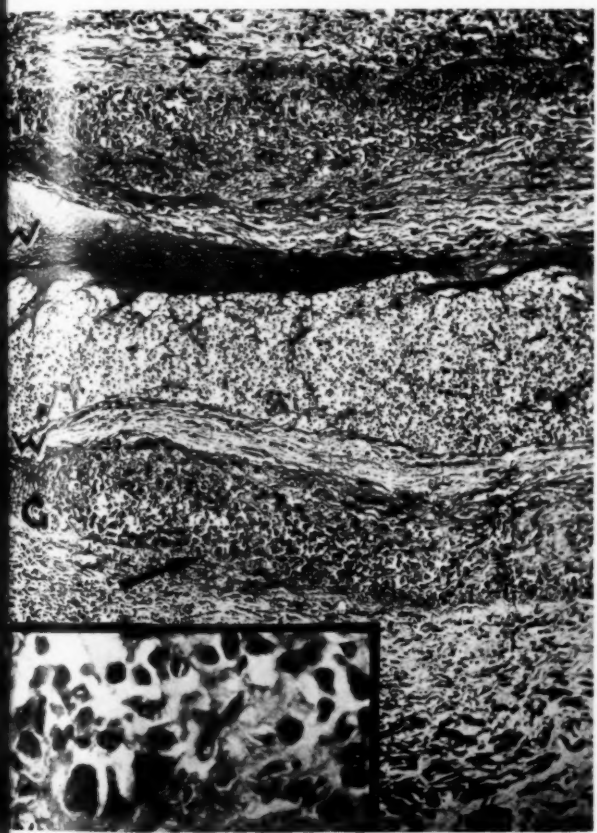


FIG. 9.—Section of the tibial artery of same subject as in Fig. 1 showing, left, tuberculoid granuloma in the intima filling a gap in the internal elastic lamella with patches of fibrin inside. Giant cells = G.C. Fibrin patch = F.P. Haematoxylin and eosin (× 120). Section on the right shows gap in internal elastic lamella (arrows). Weigert's elastica stain. (× 120.)

FIG. 10.—Section from same subject as in Fig. 1 showing granuloma (G) developing along both sides of a thrombosed artery (W) in the myocardium in polyarteritis nodosa (× 100). This granuloma is not tuberculoid, but somewhat resembles a rheumatic nodule. See particularly the inset (× 350). Haematoxylin and eosin.

FIG. 10

The vascular granuloma in polyarteritis nodosa may thus resemble the histological picture of Buerger's disease and "temporal arteritis." Gordon and Thurber (1946) and Harrison (1948) suggest that temporal arteritis has many points in common with polyarteritis nodosa. A less tuberculoid form of granuloma may also be seen in the vessel wall, resembling more closely a rheumatic granuloma with predominantly mononuclear cells and "giant cells" with hyperchromatic nuclei. Fig. 10 shows a section of a vessel wall from a case of polyarteritis nodosa demonstrating the resemblance to the more localized rheumatic granuloma.

Discussion

It seems clear that on microscopical grounds an identical vascular lesion occurs in polyarteritis nodosa, in acute rheumatic disease, and in the pararheumatic diseases as defined above. The focal lesions outside the vessels, referred to as paravascular foci, which occasionally occur in polyarteritis, are closely similar to, if not identical with, the "reticular Aschoff body" (Gross and Ehrlich, 1934), which is probably the lesion described by Klinge as "early infiltrate." Whether or not the coronal Aschoff body (Gross and Ehrlich, 1934) arises at the site of a resolving reticular Aschoff body is still a matter for argument, as is also the question of its specificity (Siegmund, 1931). What is important for present purposes is the similarity of the paravascular focus of polyarteritis nodosa to the "early infiltrate" (reticular Aschoff body) of acute rheumatic disease.

If it be accepted that the coronal Aschoff body be different, then acute rheumatic disease has all the components which characterize polyarteritis nodosa, plus an additional lesion. Similarly, a pararheumatic disease, such as acute disseminated lupus erythematosus, may show vascular changes which resemble those of polyarteritis, with certain additional features which are specific for disseminated lupus erythematosus, if we accept the views of Klemperer (1950) and Klemperer, Gueft, Lee, Leuchtenberger, and Pollister (1950).

It is likely that the future will show the similarities to be more significant than the differences, but it is already justifiable to say that the polyarteritic lesion is an important part of the morbid anatomy of acute rheumatic disease and of the pararheumatic diseases.

Polyarteritis Nodosa and Löffler's Syndrome.—It does not appear to be certain whether polyarteritis also plays a part in the transitory pulmonary infiltrations with eosinophilia known as Löffler's syndrome. On histological grounds there would appear to be two main types of this disease. The first is the commoner form, which shows focal eosinophil-celled bronchopneumonia with eosinophil-cell infiltration of vascular walls, but without any sign of fibrinoid necrosis (von Meyenburg, 1942a and b; 1944–5), or other evidence of polyarteritis nodosa. The second is the type which shows necrotizing arteriolitis and phlebitis, associated with granulomata, sometimes referred to as "rheumatoid" or "tuberculoid"; these are either necrotic or fibrosing and contain eosinophils or oxyphilic granular debris, and areas of fibrinoid swelling of collagen (Bergstrand, 1946; Baggenstoss *et al.*, 1946; Lumb, 1950). Most of the cases of this type which have been recorded had bronchial asthma during life and showed characteristic anatomical changes.

In sharp contrast to these, two personally observed cases showed not only the concomitant presence of eosinophil-cell granuloma and eosinophil-cell alveolitis, but also vascular changes which, particularly on the basis of the gaps in their elastica, were considered to be examples of burnt-out polyarteritis nodosa.

Case 5.—A man, aged 47, had generalized exfoliative dermatitis and recurrent erythroderma for two years, chiefly involving the feet, legs, arms, and scrotum. An excised inguinal lymph node showed the so-called "lipomelanic reticulosis." There was a raised serum globulin and a blood eosinophilia of 9–10%. Death was due to a sudden cerebral haemorrhage. At necropsy it was seen that there was extensive cerebral haemorrhage of the right parieto-occipital region, dermatitis, superficial ulcers on the posterior wall of the larynx, fatty liver, and a few renal infarcts. The lungs were emphysematous with ill-defined areas of lobular consolidation, notably in the lower lobes. No plugging of the bronchi was seen. The naked eye changes of the lung were in no way characteristic.

Histological examination showed that eosinophils were present in many organs. On the tongue they formed small foci eroding the thin epithelial lining from below. They were present in the deeper strata of the laryngeal ulcers, in a post-mortem clot, in myocardial septa, and in the spleen (pulp and centres of Malpighian corpuscles). The main changes were in the lungs, and were as follows.

(*a*) Multiple *nodules* in the interlobular septa: some of these were purely fibrotic and attached to unchanged vessels, others consisted of soft, oedematous, and fibrosing ground tissue.

(*b*) Collections of *eosinophil cells*: these were present as collections in diffusely thickened septa around dilated vessels (Fig. 11 EC) and as masses forming the necrotic centres of the soft fibrotic nodules mentioned above (Fig. 12).

(*c*) Vascular changes: in the larger arteries the intima was generally thickened with *concentric fibrotic cushions* containing a large number of eosinophil cells, some of which formed a subendothelial fringe. There were definite and fairly extensive gaps in the internal elastic lamella, adjacent to and opposite the intima cushions (Fig. 13). Gaps in the internal elastic lamellae were also found in some completely obliterated arteries (Fig. 14). The hilar lymph nodes contained fibrotic areas with concentric hyaline thickening of small vessels, without distinctive features.

Case 6.—A man, aged 46, had had dyspnoea for eight months, and a scaly, papular rash, not unlike pemphigus vegetans, on face, neck, and one arm, with erythroderma and exfoliation.

At necropsy the lungs were found to be emphysematous, with mucous intrabronchial plugs, associated with gross cyanosis and dilatation of the right heart.

The main histological changes were in this case also found in the lungs.

(*a*) Intrabronchial changes, typical of bronchial asthma, namely whorled plugs of mucus containing many eosinophil cells and some Charcot–Leyden crystals, were seen. There were also concentric and regular warty thickenings of the bronchial mucosa around cores of conglutinated elastic fibres.

(*b*) Necrotizing eosinophil cell alveolitis forming well defined nodules with a well preserved alveolar elastic pattern (Fig. 15) was found. The cells filling both alveolar spaces and septa were almost exclusively eosinophils. Occasionally a fibrinoid-necrotic patch could be seen lining a dilated alveolar duct on one side, with eosinophils and what appeared to be desquamated and confluent alveolar epithelium (Fig. 16).

(*c*) Vascular changes affected small arterial branches, and included fibrous intimal cushions filled with eosinophil cells and concentric fibrous thickening of the walls with gaps in the internal elastic lamellae (Fig. 16, inset).

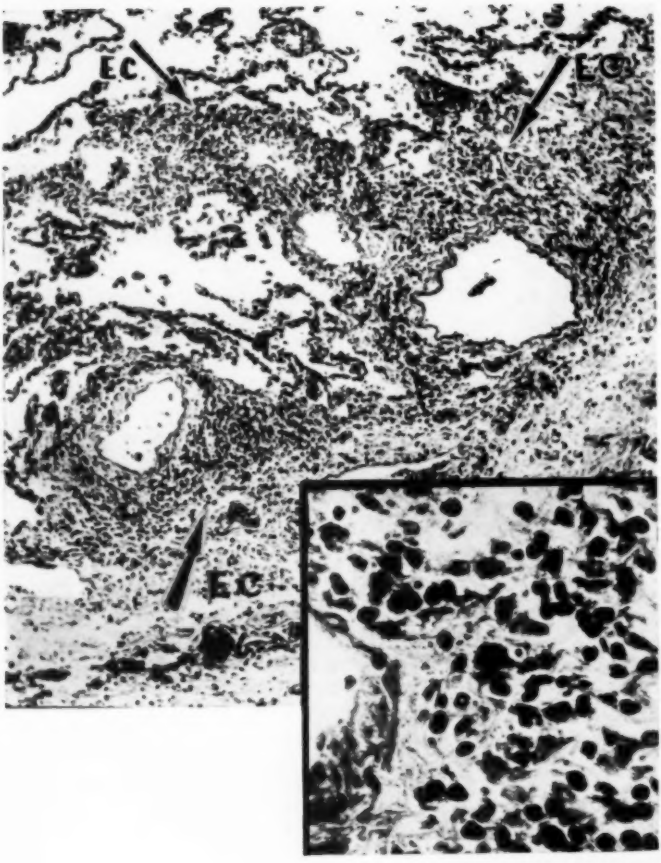
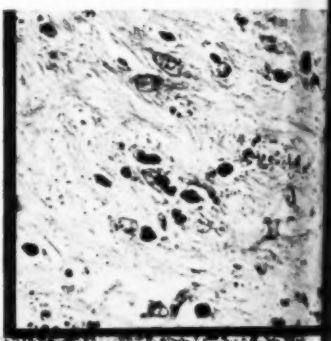


FIG. 11.—Section of lung from Case 5 showing cuffing of pulmonary vessels by eosinophil cells EC (× 100). Inset: high-power view (× 350).



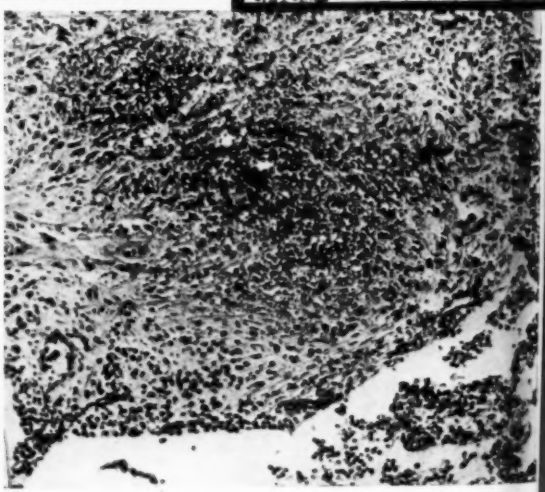


FIG. 12.—A second section of lung from Case 5 showing eosinophil-cell nodule in an interlobular septum of the lung (× 120), and necrotic centre full of eosinophil cells and scattered eosinophil granules (× 300).

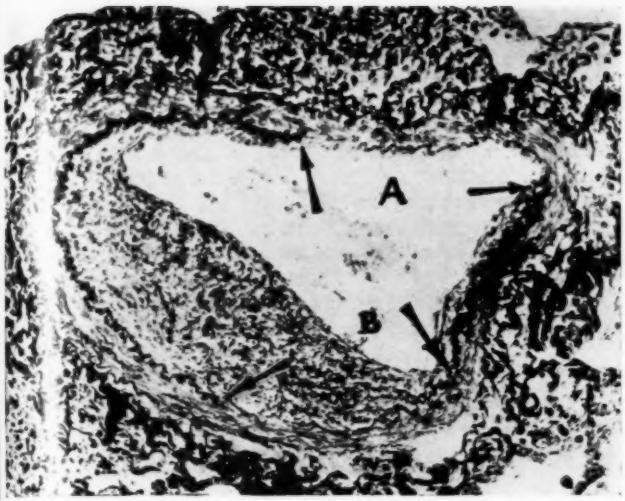


FIG. 13

FIG. 13.—Section from Case 5 showing eccentric fibrotic cushion of the arterial intima in the lung. Elastic stain showing gaps in the internal elastic lamella at A and B (× 120).

FIG. 14.—This section also from Case 5 shows the gap (G) in the elastic lamellae of the wall of an obliterated branch of the pulmonary artery (arrows). Elastic stain (× 80).

FIG. 14

These two cases show the unusual combination in the lung of eosinophil-cell granuloma with healed polyarteritis nodosa, associated with bronchial asthma in one patient and with a dermatitis in both patients. Histologically, the picture may be described as a " dissociation " of vascular changes and eosinophilia. Eosinophil-cell infiltration appears to be an independent phase rather than a reactive by-product of polyarteritis nodosa.

The definition of Löffler's syndrome is rather vague, but the observations made on these cases certainly indicate that they belong to the group described by Baggenstoss *et al.* (1946). The early polyarteritis present in the group of cases of pulmonary eosinophil granuloma following asthma suggests that the aetiology of the condition may be associated with polyarteritis nodosa. It may be recalled that polyarteritis with dermatitis or eosinophilia has been reported in association with hypersensitivity to iodine (Rich, 1945) and to dilantin sodium (van Wyk and Hoffmann, 1948). An example of fatal iodine sensitivity following bronchography (to be published by Sumner, Lichter, and Nassau) showed extensive exfoliative and bullous dermatitis with ulcerative bronchitis and gastritis. The arterioles related to the areas of ulceration, and a few arterioles elsewhere showed foci of eccentric mural necrosis with gaps in the internal elastica, a picture very like that of polyarteritis nodosa.

Pathogenesis and Aetiology of Polyarteritis Nodosa.—The disease is histologically characteristic, especially when taken as a whole. It is true that some of its features can be seen in essential hypertension, but in this disease the usual change is concentric hyaline swelling of the arterial wall, with fibrosis, in contrast to the eccentric swelling, fibrinoid necrosis, and perivascular collection of cells, notably eosinophils, seen in polyarteritis nodosa. Hypertensive changes tend to show a predilection for certain organs ; those in polyarteritis nodosa do not. The changes in vessels involved in a chronic inflammatory or ulcerative process can, at a certain stage, resemble polyarteritis nodosa, but the lesions are restricted to the vessels in the affected area.

There are a number of conditions in which the changes of polyarteritis nodosa do not appear to be accidental or secondary but form an essential component. This fact alone suggests that the changes are indicative of a tissue response to a variety of stimuli rather than to one single agent. Evidence has been adduced, both in man and the experimental animal, that a hyperergic antigen-antibody reaction may produce the characteristic lesions. Such observations have been made in allergic persons (asthmatics) suffering from serum sickness or reacting to a course of chemotherapy, notably with sulphonamides (Rich, 1942 ; Rich and Gregory, 1943 ; Black-Schaffer, 1945 ; Rosenak and Maschmeyer, 1945 ; van Rijssell and Meyler, 1948). In the experimental animal the changes at the site of local serum hypersensitivity (Arthus phenomenon) typically include eccentric fibrinoid necrosis of vascular walls with cell collections (Pagel, 1939). In the experimental reproduction of polyarteritis nodosa serum-antigens are normally used (Rich and Gregory, 1943 ; More and McLean, 1949).

The natural history of polyarteritis nodosa and related diseases justifies the search for bacterial antigens. Experiments have been carried out in human volunteers with intradermal injections of heat-killed streptococci (Humphrey and Pagel, 1949). At the site of injection in normal persons there were minimal changes after 10 to

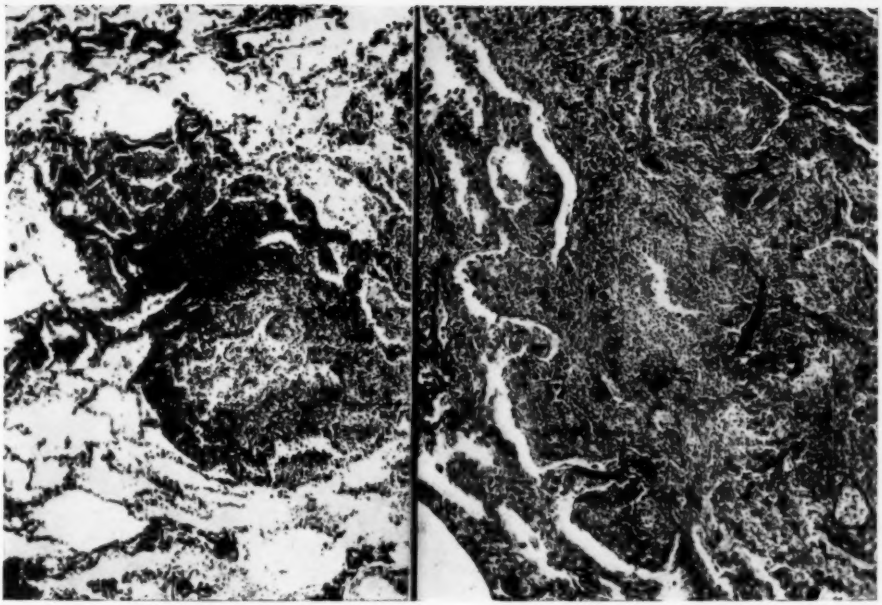


FIG. 15

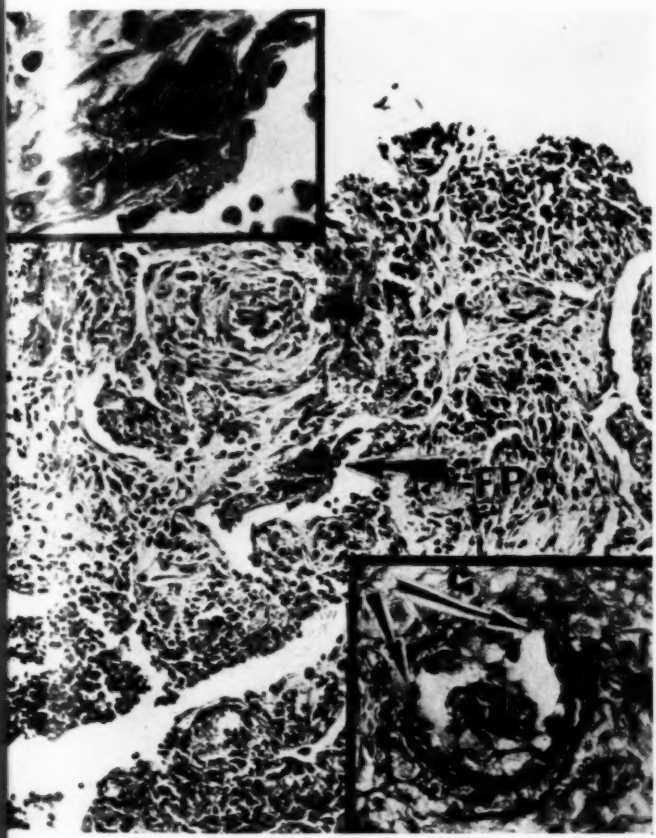
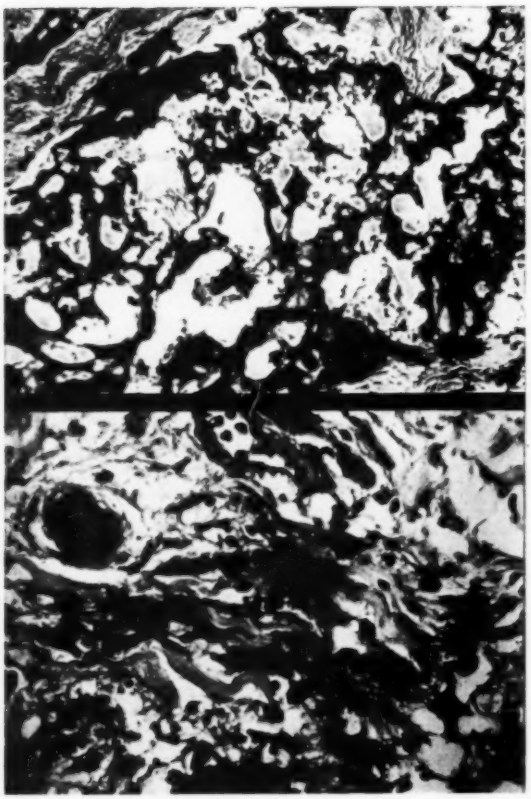


FIG. 15.—Section of lung from Case 6 showing nodular eosinophil-cell alveolitis with the alveolar elastic pattern preserved. Left: haematoxylin and eosin (× 80). Right: elastin (× 120).

FIG. 16.—Second section from Case 6 showing fibrinoid patch bordering the wall of dilated alveolar duct (F.P.) haematoxylin and eosin (× 120). Inset (top): The fibrinoid patch (× 350 haematoxylin and eosin). Inset (bottom): Arteriole with gap in internal elastic lamellae. Elastic stain. (× 350.)

FIG. 16

14 days, with perivascular collection of a few lymphocytes and macrophages. Numerous cocci were seen in the macrophages, indicating the disposal, via the lymphatics, of the bacterial foreign body. The results were quite different in patients with rheumatic fever and subacute bacterial endocarditis. In these diffuse oedema was prominent in the early stages, and later, in the more severe type of response, oedema was patchy and focal with fibrin exudation, conglutination of collagen fibres, and loss of staining affinities. Pictures thus obtained were indistinguishable from the infiltrative changes characteristic of early rheumatic fever (Figs. 4 and 5) and lupus erythematosus disseminatus. Figs. 17 and 18 compare the changes following intradermal injection of heat-killed streptococci in a rheumatic fever patient, aged 21, with those of an early rheumatic nodule in the skin of the hand of a patient aged 33 years. Focal oedema and fibrinous conglutination of collagen fibres are present interstitially (Fig. 17) and the cells are swollen and vacuolated. There is a tendency for the cells to group themselves around patches of fibrin and conglutinated ground substances, which act as foreign bodies and produce a surrounding granulomatous reaction, with the formation of giant cells. There is eccentric fibrin infiltration and necrosis of the vascular intima (Fig. 18). No cocci were seen in any of these lesions.



An example has therefore been shown of the production of the so-called "anaphylactic" type of hypersensitivity with oedema and fibrinoid necrosis of vascular walls following the injection of a bacterial antigen. This anaphylactic type of hyperergic response is usually attributable to serum hypersensitivity and distinguished from the delayed necrotic changes of the "tuberculin type" observed in bacterial hypersensitivity. It is probable that the bacterial antigens produce a "trigger action," and the results may possibly be due to antigens which are common to bacterial and serum proteins.

FIG. 17.—Top: Section of acute rheumatic skin nodule from male aged 33 years showing focal oedema and fibrin infiltration of collagen. Phosphotungstic acid-haematoxylin stain. Bottom: Section from human volunteer aged 21 years with rheumatic fever showing focal oedema and fibrin infiltration of collagen 18 days after intradermal injection of heat-killed streptococci. Haematoxylin and eosin (× 250).

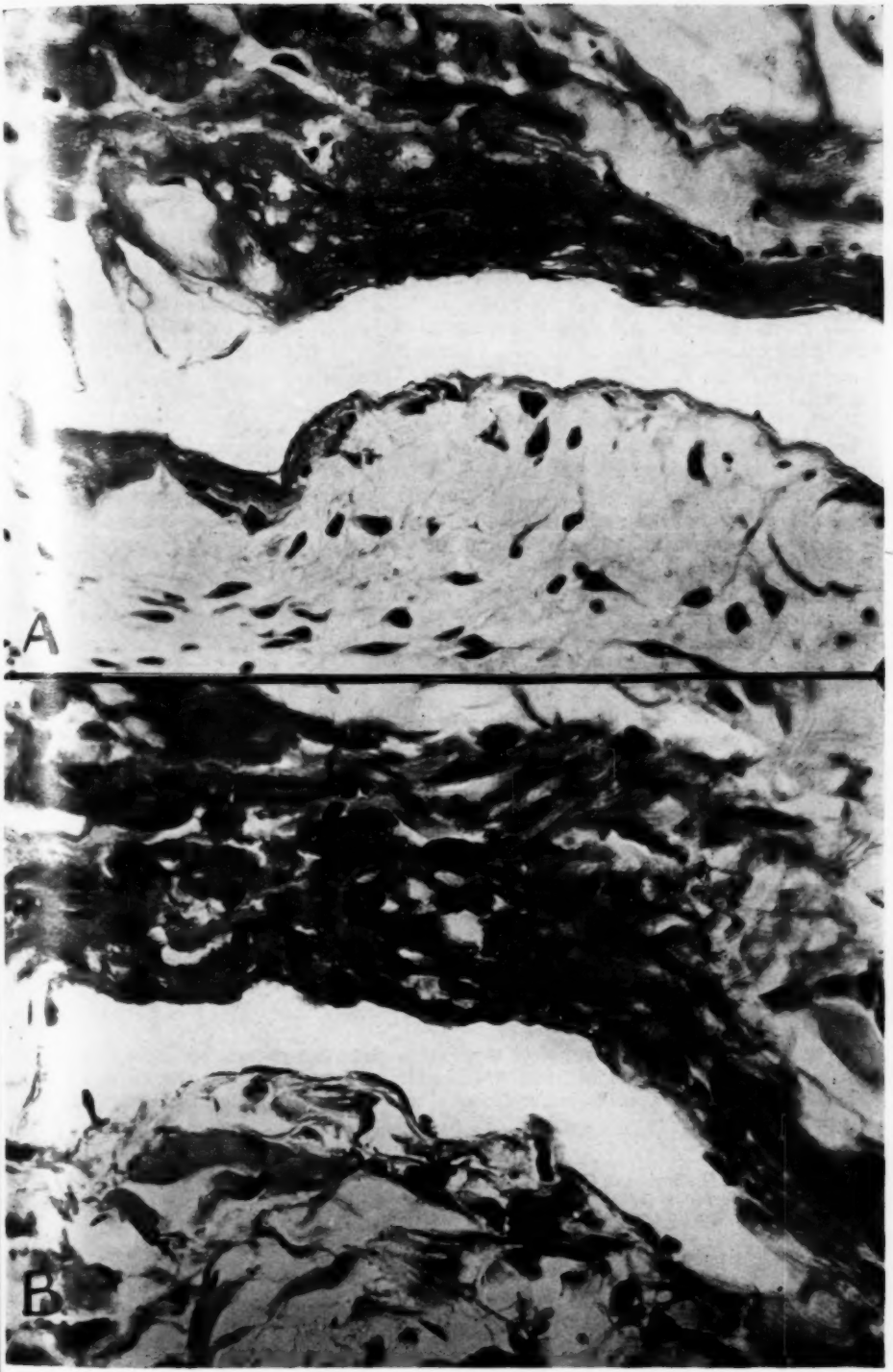


FIG. 18.—Same subject as top Fig. 17 showing eccentric oedema and fibrin infiltration of intima. Haematoxylin and eosin (× 450). Bottom: Same subject as in Fig. 17 (bottom) showing the same change induced by intradermal injection of heat-killed streptococci. Haematoxylin and eosin (× 450).

These experimental results in the animal and in human volunteers suggest a multiplicity of aetiological agents which may produce polyarteritis nodosa and the related conditions, the common pathogenic factor being antigenic hypersensitivity which is expressed in common tissue changes.

Summary and Conclusions

The early changes in polyarteritis nodosa appear to be subendothelial swelling due to permeation of fluid, followed by fibrinogen exudation with subsequent—mostly eccentric—necrosis of the vascular wall. A breach in the latter can be plugged by fibrin, or fibrin may be found both inside and outside the breached and necrotic vascular wall. The term "fibrinoid" should be restricted to the deposited material that fails to stain like fibrin.

The differences between polyarteritis nodosa and the rheumatic and "para-rheumatic" diseases (lupus erythematosus disseminatus, dermatomyositis, "arteriolitis allergica," temporal arteritis) concern the natural history of these diseases and the site and distribution of the lesions, but the basic histological change appears to be identical in all. In polyarteritis nodosa, fibrin deposition and necrosis affect the vascular walls mainly, but extramural foci may occur and involve the collagen fibres, with lesions indistinguishable from the acute "infiltrative" changes in rheumatic fever, lupus erythematosus disseminatus, and dermatomyositis. There is characteristic preservation of the argyrophil reticulin within the zone of collagen change. In addition to these paravascular foci, pseudo-tuberculous necrotic lesions may occur in polyarteritis nodosa. Finally, granuloma may develop in the vascular wall, resembling the Aschoff nodule and the changes seen in temporal arteritis. On the other hand, "rheumatic arteritis," as typically seen in rheumatic fever, can be indistinguishable from polyarteritis nodosa and the vascular changes observed in dermatomyositis and scleroderma.

Two cases are described of eosinophil-celled alveolitis (one form of Löffler's syndrome) in association with exfoliative dermatitis and asthma. In both there is evidence of burnt-out polyarteritis nodosa and of a "dissociation" of eosinophilia and arterial necrosis.

The course of events which leads to the explosive arterial lesion of polyarteritis nodosa is indicative of hyperergy. This hypersensitivity may be to a foreign or modified serum, or to abnormal protein, being comparable to the Arthus phenomon and of the type generally referred to as the "anaphylactic type of allergy." On the other hand the hypersensitivity may be to a bacterial antigen, as shown by the use of streptococci in experiments on human volunteers.

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THE HAEMAGGLUTINATION REACTION IN TUBERCULOSIS

BY

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Circulating antibodies reacting with tubercle bacilli or their extracts have been demonstrated repeatedly in the sera of patients suffering from pulmonary and other forms of tuberculosis. In a review of the subject Wilson and Miles (1946) suggest that a test based on the agglutination of tubercle bacilli is unreliable and that the precipitin test made by mixing the patient's serum with tuberculin is of no practical importance. The complement fixation test in the hands of various workers gave positive results in from 38 to 90% of patients with different forms of the disease, but it is also positive in from 10 to 15% of healthy persons and in those suffering from syphilis, leprosy, and malaria.

Middlebrook and Dubos (1948) showed that sheep's red cells suitably treated with an extract of the H 37 strain of *Myco. tuberculosis* were agglutinated by the sera of six rabbits immunized with the B.C.G. strain; titres up to 1,280 were obtained. The cells were also agglutinated by the sera of six patients with active tuberculosis of the lungs to titres of 8 or over, and one patient with miliary disease gave a titre of 256. The sera of two Mantoux-negative nurses agglutinated the cells to a titre of 4; the cells were not agglutinated by miscellaneous antibacterial sera with the exception of a pneumococcus type XIV antiserum, which reacted only to a titre of under 32. Wassermann-positive sera did not react with the cells. A specimen of "old tuberculin" was also found to be capable of sensitizing sheep's red cells in a similar way, and therefore it appeared that at least one component of the "antigen" was heat stable. Evidence was also presented that the sensitizing substance or substances might be associated with the polysaccharide fraction of the bacillary extract. The same technique was used by Gernez-Rieux and Tacquet (1949), who examined the sera of 95 tuberculous patients and 43 controls. Of the tuberculous patients 80% gave titres of 8 and over, while 30% of the controls gave titres of 2 or 4 but none of 8 or over. With minor modifications the method has been tested by various workers. Sohier (1950) used a purified tuberculin for the sensitization of red cells. He appears to have obtained satisfactory results with tuberculous sera, but found false positives in his control series. He mentioned the use of human cells instead of sheep cells for the test, but did not carry out large-scale investigations. Smith and Scott (1950) used Lederle tuberculin concentrated four times for the sensitization of sheep's cells and found that 80% of 104 tuberculous patients gave positive reactions at titres of 2 and

upwards ; three out of 62 tuberculin-negative controls gave positive results. They also tested the sera of 58 students before Mantoux testing. Of these 21% gave positive results. After Mantoux testing the sera of the students were re-examined, and this time 49% of them were found to be positive. They concluded that the test was mainly positive in active tuberculosis. Rothbard, Dooneief, and Hite (1950) examined 168 sera from patients with active tuberculosis. Of these 92.3% were positive at titres of 8 and over. None of 216 control sera was positive at that level. Of the control sera, however, 6% gave agglutination at lower titres.

For the purposes of the present investigation it was thought that the use of human Group O red cells, preferably Rh-negative, instead of sheep's cells, would simplify the technique since inactivation and absorption of the individual sera would no longer be necessary. The incidence of natural anti-O agglutinins in human sera is estimated to be of the order of 1 : 1,000 (Wiener, 1943) ; this remote contingency was covered by the use of a single control tube in which unsensitized cells were mixed with the highest concentration of the serum employed. Other modifications included the use of a lightly buffered saline solution as a diluent for the sensitizing agents tested and for the washing and dilution of the red cells in order to maintain the reaction of the various reagents approximately neutral. Chown's capillary tube method (Discombe and Meyer, 1948), adapted to a quantitative estimation of the haemagglutinative titre of patients' sera, was found to be most serviceable and economical in glassware and labour.

Methods

Buffered Saline.—Solution (a): 10.207 g. of anhydrous potassium dihydrogen phosphate were dissolved in 500 ml. of distilled water.

Solution (b): 53.73 g. of anhydrous disodium phosphate were dissolved in 1 litre of distilled water.

Then 389 ml. of solution (a) were added to 611 ml. of solution (b) to make 1 litre of isotonic 0.15 M buffer, *p*H 7.0. One volume of this buffer was then mixed with 9 volumes of saline (0.9% sodium chloride), giving a final molar concentration of buffer of 0.015.

Preparation of Sensitizing Agents.—Those used were "crude carbohydrate fraction," glycerol extracts, and tuberculin P.P.D.

"Old Tuberculin."—Burroughs Wellcome "old tuberculin" was used, and was found to have a *p*H of 8.4 and to be hypertonic, containing 5.13 g. of sodium chloride per 100 ml. It was therefore diluted before use by the addition of 5 volumes of distilled water, the reaction being adjusted to *p*H 7.0 with N/1 hydrochloric acid ; 10 ml. of this mixture were used for the treatment of 0.5 ml. packed red cells.

"Crude Carbohydrate Fraction" (C.C.F.).—A partial fractionation of three different "old tuberculins" was carried out in order to produce protein-free solutions of polysaccharide for the sensitization of red cells. The method was similar to that described by Wieghard and Julianelle (1935) for preparing staphylococcal polysaccharides.

Tuberculin, 10 ml., was mixed with 10 ml. of distilled water and 20 ml. of 50% trichloracetic acid were added. The mixture was well shaken and allowed to stand about one hour until the precipitate became flocculent. The latter was separated by centrifugation and the clear brownish supernatant mixed with 6 volumes of 95% commercial ethanol which had been saturated with sodium acetate. This mixture was well shaken, stood for an hour in the cold, and the granular white precipitate filtered

off or centrifuged. The precipitate was dissolved in the minimum amount of distilled water (about 2 ml.) and tested for the presence of protein by the addition of a little 50% trichloracetic acid: none was found at this stage in any batch. The watery solution was again mixed with 6 volumes of ethanol, the resulting precipitate spun down in a 50-ml. centrifuge tube, shaken with 40 ml. of acetone, recentrifuged, and the supernatant liquid discarded. The average dry yield from 10 ml. of old tuberculin was about 0.09 g. For use in the tests the precipitate was not dried, but was dissolved in 20 ml. of buffered saline and gently warmed until the smell of acetone had gone. A quantity of 10 ml. of this colourless opalescent liquid was used for the treatment of 0.5 ml. of packed red cells.

The tuberculins from which these crude fractions were obtained were (*a*) Burroughs Wellcome "old tuberculin," derived from the human strain H 37 Rv; (*b*) Evans "old tuberculin" from N.C.T.C. 276 (human); (*c*) Evans "old tuberculin" from N.C.T.C. 913 (bovine).

The fractions from these different strains appeared similar in behaviour and were all capable of sensitizing human red cells. Most of the work was done with the fraction obtained from (*a*) and will be referred to as C.C.F. (H 37).

Glycerol Extracts.—Bulk lots of tubercle bacilli, grown on the surface of a fluid synthetic medium at 37° C. for ten weeks and killed by steaming for three hours, were obtained as a by-product from the manufacture of "Weybridge P.P.D. tuberculin" (Green, 1946). One hundred grammes (moist weight) of bacilli were added to a flask containing 1 litre of a 5% solution of glycerol in distilled water; the flask was then heated at 100° C. until the contained fluid had evaporated down to a volume of 100 ml. A portion of the grumous mixture resulting from this concentration was diluted with an equal volume of distilled water, and to this mixture an equal volume of 50% trichloracetic acid added. This mixture was shaken, allowed to stand for 15 minutes, and centrifuged; the deposit consisted of undissolved organisms and precipitated protein. The supernatant liquid was mixed with 95% commercial ethanol and the rest of the process described under the heading "crude carbohydrate fraction" carried out, in order to obtain a carbohydrate fraction comparable to that from the commercial "old tuberculins."

Four strains were treated in this way, a separate extract being made from each strain. They were human strains "DT," "PN," and "C" (Bureau of Animal Industry, Washington, U.S.A.), and an avian strain.

Tuberculin P.P.D.—The diagnostic tablets of tuberculin P.P.D. Parke Davis used for intradermal Mantoux testing were dissolved in 2 ml. of buffered saline to give a concentration of 0.001 g. per ml., and this solution was used for the sensitization of 0.1 ml. of packed red cells for the testing of a small number of sera.

Sensitization of Red Cells.—Ten ml. of citrated human Group O Rh-negative (D−) blood were placed in a 30-ml. screw-cap universal container and the red cells spun down. The supernatant plasma was removed, and the cells washed three times with 20-ml. quantities of buffered saline. These centrifugations were done in an angle centrifuge at 2,000 r.p.m. for five minutes. Half a millilitre of the packed red cells was pipetted into a centrifuge tube containing 10 ml. of the sensitizing agent, and mixed by inversion two or three times. The mixture was incubated in the water bath at 37° C. for two hours, being mixed at intervals. The cells were then spun down, the supernatant discarded, and the cells washed again three times to remove all traces of the sensitizing substance in the suspending fluid. The packed cells from the third washing were mixed with four times their volume of buffered saline to give just over 2 ml. of a roughly 20% suspension, sufficient to test about 50 sera.

A 20% suspension of unsensitized cells was also made for use as a control in the testing of each serum.

Agglutination appeared to be most rapid and clear-cut when the citrated human blood used for the preparation of cell suspensions was kept no longer than three weeks in the refrigerator, and when the sensitized cells were used immediately after they had been prepared. Sensitized cells kept in the cold for one night and washed once before use gave satisfactory results, but after this time were discarded. Red cells from four individuals were used, and no differences in their reactions were observed.

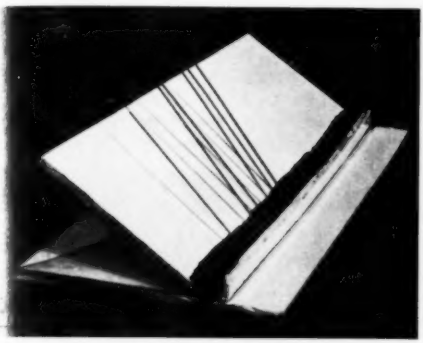

FIG. 1*a*.—The holder upright.

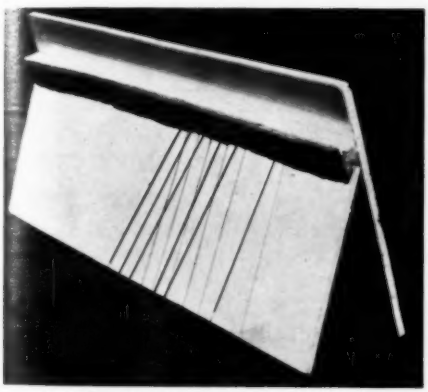

FIG. 1*b*.—The holder inverted.

FIG. 2.—Appearances of the completed test in two sera. The set of five tubes on the left shows agglutination in the first three tubes (1 : 2 = ++, 1 : 4 = ++, 1 : 8 = +, 1 : 16 = negative). Control tube is negative. The set on the right is negative in all five tubes.

Testing of Sera.—Doubling dilutions in buffered saline of about 0.2 ml. of serum were made on a glazed china blood-grouping tile, starting with 1 : 2 and ending with 1 : 16. Sera agglutinating to a titre of 16 were usually retested in higher dilutions. Batches of three sera could thus be diluted on a single tile.

Testing was carried out by Chown's capillary method as described by Discombe and Meyer (1948) using capillary tubes 10 cm. long with a bore of 0.4 to 0.7 mm. For each serum, a capillary

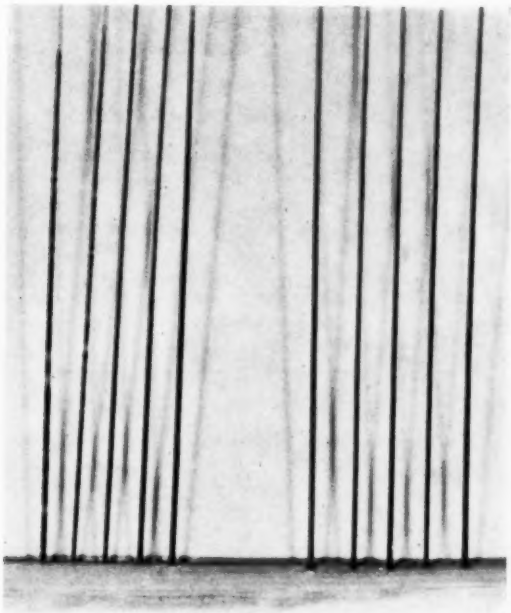

FIG. 2

tube was first dipped into the suspension of *unsensitized* cells until the latter had run up about 2 cm., and then into the first serum dilution (1 : 2) until the capillary contained a 9-cm. column of liquid. This tube served as a control against the possible (though very unlikely) presence in a serum of " cold " agglutinins or iso- or immune-agglutinins against the natural blood group antigens of the test cells. The test proper was done in the same way using sensitized cells. The lower end of each capillary was stuck at an angle of 45° into a strip of plasticine mounted on a holder made of white perspex which could be stood upside down without disturbing the tubes (Fig. 1). The holder, complete with capillaries, was placed in the 37° C. incubator, and after about eight minutes, the time required for the first sedimentation of the red cells, it was inverted. The readings were taken after a quarter of an hour's incubation, agglutination being indicated by delayed sedimentation together with a characteristic and unmistakable segmentation of the column of cells: a negative result was noted when the cells fell rapidly, leaving a smooth unbroken " tail " above the main mass (Fig. 2). Further incubation up to an hour, with repeated inversion of the holder, showed no increase in titre with 10 positive sera of different titres and was not therefore carried out as a routine.

Experimental Findings

Altogether 471 sera, 223 from tuberculous patients and 248 from controls, were tested. None agglutinated the control unsensitized cells, nor was suspension-instability of sensitized cells found to occur.

" Old Tuberculin " as a Sensitizing Agent.—The sera from 66 patients with pulmonary tuberculosis and 89 hospital in-patients admitted for other conditions were tested using cells sensitized with " old tuberculin " (Burroughs Wellcome), and the results are shown in Table I. The difference between the two sets of patients is statistically significant, and when the higher titres are considered the significance

TABLE I

AGGLUTINATION TITRES OF 155 HUMAN SERA USING OLD TUBERCULIN (H 37)

Sera	Number Tested	Negative	Positive at Serum Dilutions			
			1/2	1/4	1/8	1/16 or Over
Tuberculous ..	66	15 (23%)	19 (29%)	14 (21%)	11 (17%)	7 (10%)
Control	89	51 (57%)	27 (31%)	8 (9%)	—	3 (3%)

is greater. However, about one-third of the control sera were positive at a titre of 2 and some at 4. The results obtained suggest that agglutination of the sensitized red cells is due to antibodies to the tubercle bacillus but that other non-specific factors may also cause the reaction. Thus a test based on this technique and using this " old tuberculin " is of little value for diagnosis.

The Role of Tuberculo-proteins.—Since the majority of the control patients referred to in Table I were Mantoux-positive, the possibility was considered that the false positive findings might be due to the action of a circulating antibody against the tuberculo-proteins responsible for the tuberculin reaction. An attempt

was therefore made to sensitize red cells with the purest available tuberculo-protein, namely "tuberculin P.P.D." Twenty-three sera from non-tuberculous individuals were tested, of whom 12 were Mantoux-negative and 11 Mantoux-positive. Since few of the sera in this series reacted at 1 : 2 dilution, they were tested undiluted. These sera were similarly tested against cells sensitized with C.C.F. (H 37) and a fair agreement was found in both groups: 10 out of 13 sera of the Mantoux-negative group failed to react with both kinds of sensitized cells, and eight out of 10 in the Mantoux-positive group agglutinated both types of cells. This suggests that the reaction obtained with P.P.D. cells is due to carbohydrate impurities in the sensitizing agent. Carbohydrates are known to be present as impurities to an appreciable extent even in this purified tuberculin (Green, 1946). Thus, tuberculo-proteins do not appear to contribute to the agglutinability of these red cells. Moreover, since some of the sera of Mantoux-negative individuals gave agglutination when undiluted, the testing of undiluted sera was felt to serve no useful purpose and was discontinued.

Preliminary Tests with a Polysaccharide Fraction.—A larger series of tests was carried out using cells which had been treated with the crude polysaccharide fraction (C.C.F. H 37). Two hundred and ninety-five sera were tested, and the results are shown in Table II. These sera were again obtained from patients suffering from

TABLE II

AGGLUTINATION TITRES OF 295 HUMAN SERA USING C.C.F. (H 37)

Sera	Number Tested	Negative	Positive at Serum Dilutions			
			1/2	1/4	1/8	1/16 or Over
Tuberculous ..	134	58 (43%)	31 (23%)	20 (15%)	12 (9%)	13 (10%)
Control	161	148 (92%)	4 (2.4%)	5 (3%)	1 (0.6%)	3* (2%)

* Two of these patients were later found to be tuberculous; the third could not be followed up.

pulmonary tuberculosis, the controls being hospital in-patients with other diseases. Control and "positive" sera were tested in a random way to obviate any bias on the part of the observer. The table shows that there is an even greater contrast between the two groups of patients than in Table I, as nine-tenths of the control sera were negative at 1 : 2 dilutions, whereas over half of the tuberculous were positive at this level. Again, the difference between the groups is accentuated in the higher dilutions. As a result of these serological findings the clinical state of the three control patients giving agglutinin titres of 16 was investigated, two tuberculous lesions being found. One man, admitted for operative correction of strabismus, had a tuberculous cavity at the right lung apex, of low-grade activity. The second patient had long-standing sacro-iliac tuberculosis. The third patient had unfortunately been discharged after minor surgery and has not since been available for follow-up.

Three points were noted at this stage: first, that sera in the control series taken from the Wassermann rack after inactivation were usually completely negative;

secondly, that control sera tested very soon after taking gave an unduly high proportion of positive results, though to low titres only ; and thirdly, following this line of thought, that a few of the known positive sera showed no appreciable loss of titre after heating at 56° C. for as long as 75 minutes. It seemed possible that there might be a heat labile component in human sera producing non-specific agglutination.

The Effect of Heating Sera.—Each of 25 untested sera from tuberculous patients and of 25 control sera were divided into two portions of which one was heated at 56° C. for 30 minutes. Table III shows that, when both portions of all the sera

TABLE III

COMPARISON OF AGGLUTINATION TITRES OF UNHEATED AND HEATED SERA

		Negative	Positive at Serum Dilutions			
			1/2	1/4	1/8	1/16 or Over
25 sera from tuberculous patients	Unheated	—	1 (4%)	4 (16%)	6 (24%)	14 (56%)
	Heated	2 (8%)	11 (44%)	7 (28%)	1 (4%)	4 (16%)
25 sera from control patients	Unheated	16 (64%)	2 (8%)	5 (20%)	2 (8%)	—
	Heated	20 (80%)	5 (20%)	—	—	—

were tested with the same batch of cells treated with C.C.F. (H 37), the number of false positives fell by nearly half, and none were observed at titres higher than 2 in this small control group. There must also have been a non-specific thermo-labile element in a proportion of the tuberculous sera, since there is some fall in their titre after heating. However, the difference between the control and the tuberculous groups seems to be still further accentuated by heating.

The Findings with Freshly Drawn Sera.—Another anomaly observed was that when approximately half of a batch of tuberculous sera were tested on the day of taking and the remainder tested two days later, after storage at just above freezing-point, the second group gave lower agglutination titres and a higher proportion of negative results than was likely to be due to an error of sampling: it seemed that an appreciable deterioration of the sera had occurred with keeping in the unfrozen state. Tests were carried out based on the above findings. One hundred and twenty-six sera were investigated, 66 from tuberculous patients substantially similar to those in the various tests above, and 60 controls. Twenty-three of the latter were from hospital in-patients considered to be free from tuberculosis, and 37 were from nurses and medical students, clinically and radiologically healthy. After the minimum time had been allowed for the separation of the clot, each serum was immediately placed in the freezing compartment of a refrigerator. Tests using cells treated with C.C.F. (H 37) were immediately preceded by heating and thawing of the sera at 56° C. for 30 minutes. The results are presented in Table IV, and arbitrary plus signs have been allotted to the serum dilutions in an attempt to indicate the clinical

TABLE IV

AGGLUTINATION TITRES OF 126 FRESH HUMAN SERA (INACTIVATED) USING C.C.F. (H 37)

Sera	Number Tested	Negative	± (Positive at 1/2)	+ (Positive at 1/4)	++ (Positive at 1/8)	+++ (Positive at 1/16 or Over)
Tuberculous ..	66	5 (7.6%)	17 (25.8%)	18 (27.2%)	15 (22.7%)	11 (16.7%)
Control	60	40 (66.7%)	15 (25.0%)	5 (8.3%)	—	—

value of the test. Thus, it appears that the test would give reliable results at the 2-plus or higher levels, but the number of positives recovered is only one-third of the patients.

Correlation with Sedimentation Rates.—It was thought that these findings might be correlated with the sedimentation rates of the tuberculous patients. Fig. 3 is a "dot" diagram relating agglutination titres in 118 patients with their Westergren sedimentation rates: no correlation was observed.

Correlation with the Duration of Disease.—Another possibility was that long-standing disease might give rise to higher titres than more recent infections, and

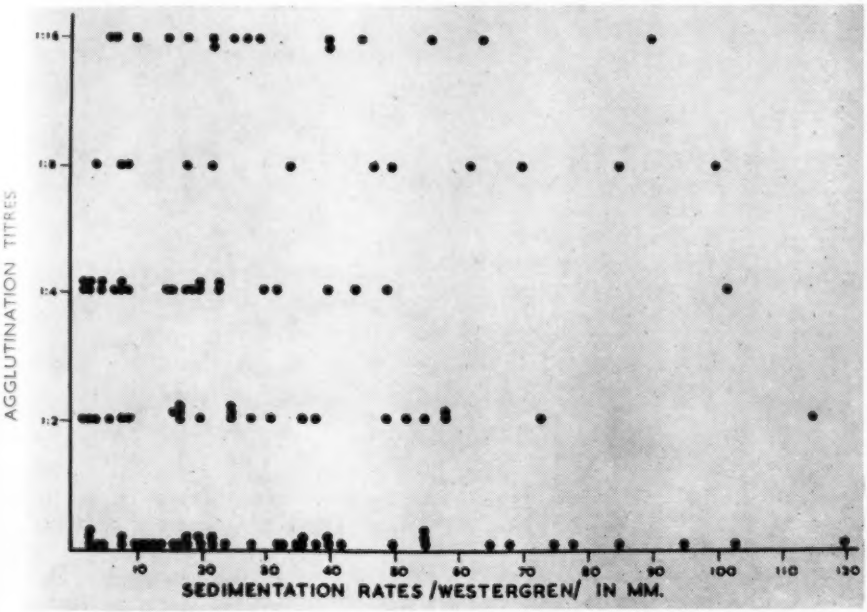

FIG. 3.—Correlation between agglutination titres and sedimentation rates.

Fig. 4 shows in "dot" form the relation between observed titres and duration of disease in 122 patients. It can be seen that higher titres were obtained with sera from those with disease of longer duration, but nevertheless there were many such that failed to show a significant titre. Most of the patients had been ill for six months to four years.

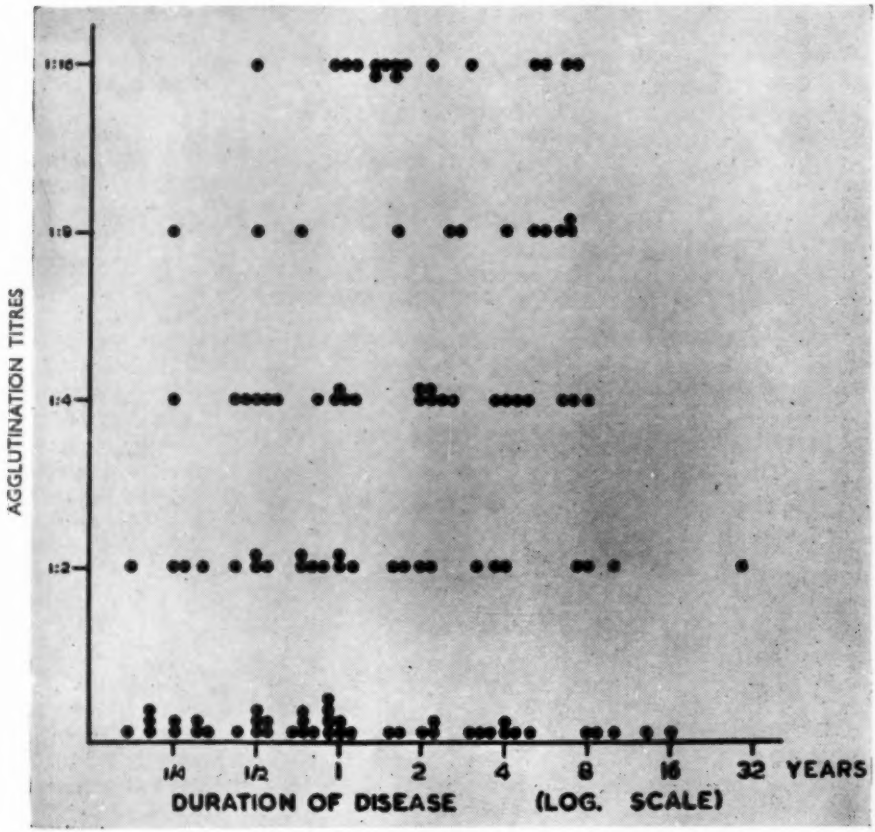

FIG. 4.—Correlation between agglutination titre and duration of disease.

The Use of Polysaccharides from Different Strains.—Since many of the sera from tuberculous patients failed to react in a significant titre (i.e., 1 : 8 or over), it seemed possible that the organism used for sensitization might not be antigenically representative of all infecting strains. The exact nature of the sensitizing agent in the crude fraction used here is not known, therefore it is impossible to assess the probability of such an antigenic difference. Similar crude polysaccharide fractions were

TABLE V

COMPARISON OF TITRES OF 61 HUMAN SERA TESTED WITH SENSITIZING AGENTS OBTAINED FROM THREE MAMMALIAN STRAINS

	Serum No.	Titres with "H 37" Cells	Titres with "NTC 913" (Bovine) Cells	Titres with "NTC 276" (Human) Cells
gglutination titres of 36 sera from tuberculous patients	526	—	—	—
	506	16	8	16
	507	8	8	8
	522	8	8	8
	502	16	8	16
	512	8	4	8
	526	4	2	4
	509	16	8	8
	505	4	4	4
	504	4	4	4
	510	16	8	16
	525	4	4	8
	523	4	2	2
	519	4	2	4
	549	4	2	4
	550	4	4	4
	537	—	—	2
	548	4	4	4
	520	2	—	2
	524	8	4	8
	544	8	4	8
	540	2	—	—
	533	4	2	2
	536	4	4	4
	521	2	—	2
	516	—	—	—
	513	4	2	4
	517	—	—	—
	514	—	—	2
	501	—	—	—
	518	—	—	—
	515	2	2	2
	503	4	2	2
	511	2	2	4
	508	16	16	16
Agglutination titres of 25 control sera	499	2	—	—
	493	—	—	—
	459	2	2	2
	451	4	4	4
	470	2	2	4
	494	—	—	—
	551	—	—	—
	462	—	—	—
	555	—	—	—
	500	—	—	—
	466	—	—	—
	557	—	—	—
	553	2	2	2
	471	2	2	4
	458	4	4	4
	554	—	—	—
	454	4	2	2
	464	—	—	—
	491	—	—	—
	453	4	2	2
	465	—	—	—
	463	—	—	—
	498	2	—	—
	495	—	—	—
	496	2	—	2

prepared from two other commercial " old tuberculins " (derived from human strain N.C.T.C. 276 and from bovine strain N.C.T.C. 913). These two extracts, and that from the H 37 strain, were used to sensitize red cells, which were then tested against 69 human sera (Table V).

In addition, three other strains of human type (" DT," " PN," and " C ") and an avian strain were extracted. These strains were obtained in the form of a wet bacterial mass, and the mode of preparation of the extracts is described in the technical methods. This technique was used because it yielded material which appeared to react in a way similar to that obtained from old tuberculins. Three of these extracts were compared with that from H 37 O.T. using 50 human sera (Table VI). The fourth extract from human strain " C," tested with a smaller number of sera, behaved similarly. It appears from these results and from Table V that none of the newer extracts was more specific than that of the H 37 or tuberculin from the point of view of a sero-diagnostic test. Furthermore, there was no obvious indication of strain variation, except that the avian extract apparently lacked the specific principle and did not render the cells susceptible to agglutination by human sera.

Discussion

As the " old tuberculin " used by the American authors was not available, the results here are not strictly comparable with their findings. It appeared reasonable to investigate the separate effects of the protein and polysaccharide constituents of tuberculin. The essentially negative findings with P.P.D. suggest that either tuberculoproteins do not adhere sufficiently to the red cells, or that no specific antibody was present in the sera tested. On the other hand, it is clear that the alcohol-precipitable fraction of protein-free tuberculin contains a specific sensitizing factor. In this connexion, it was noted that the serum of one of 16 Mantoux-negative persons was positive to a titre of 2. This finding agrees with those of Smith and Scott (1950), who showed that the serum of one out of 23 Mantoux-negative students gave a positive reaction, while after Mantoux testing five were positive. They argued that the skin-test itself stimulated antibody production, and, confirming this, found that of 58 tuberculin-positive students 12 gave positive haemagglutination reactions before, and 27 after, the Mantoux test. Positive results were also found in a small number of B.C.G.-treated individuals. It is not known whether antibody titres detected by this test appear before the skin reaction becomes positive at the time of primary infection, or if very high titres occur then: further work is needed on these points, as well as on the behaviour of the test during vaccination with B.C.G. or the vole bacillus.

Since the complement fixation reaction for tuberculosis has been found to give cross reactions with syphilitic sera, nine Wassermann-positive sera were tested by the method and found to be completely negative. This result agrees with the findings of Middlebrook and Dubos (1948) and of Rothbard *et al.* (1950), who found no higher incidence of positive reactions in syphilitic sera than in their general series of non-tuberculous controls.

It seems paradoxical that heating sera appears to increase the specificity of the test, while the keeping of sera in the unfrozen state leads to loss of potency. It is

TABLE VI

COMPARISON OF TITRES OF 50 HUMAN SERA TESTED WITH SENSITIZING AGENTS FROM AN AVIAN AND THREE HUMAN STRAINS

	Serum No.	Titres with "H 37" Cells	Titres with "DT" Cells	Titres with "PN" Cells	Titres with Avian Cells
xteen sera from tuberculous patients showing titre variation of no significance	6	2	4	4	—
	8	2	2	4	—
	9	16	16	16	—
	13	8	4	8	—
	14	4	4	8	—
	16	16	16	16	—
	19	2	4	4	—
	20	4	4	8	—
	25	4	2	4	—
	26	16	16	16	—
	27	16	16	16	—
	35	8	4	4	—
	36	—	2	2	—
	37	8	8	16	—
	39	8	8	8	—
	40	8	16	16	—
(Total 25)					
ne sera from tuberculous patients showing titre variation of doubtful significance	1	4	—	4	—
	4	8	4	16	—
	5	8	4	16	—
	7	4	16	16	—
	15	—	—	4	—
	17	4	2	8	—
	29	4	4	16	—
	32	2	8	8	—
	33	2	2	8	—
ighteen control sera showing titre variation of no significance	10	2	—	2	—
	2	—	—	—	—
	11	—	2	—	—
	12	4	4	4	—
	21	2	2	4	—
	22	2	4	4	—
	30	8	8	16	—
	31	2	4	4	—
	34	2	2	2	—
	38	—	—	—	—
	41	—	—	2	—
	42	—	—	—	—
	43	—	—	2	—
	46	—	—	—	—
	47	—	—	—	—
	48	—	—	—	—
	49	2	4	4	—
	50	—	—	—	—
(Total 25)					
Seven control sera showing titre variation of doubtful significance	3	8	4	16	—
	18	2	2	8	—
	23	4	16	8	—
	24	2	4	8	—
	28	4	4	16	—
	44	2	4	8	—
	45	—	2	4	—

known, however, that Rhesus antisera of human origin deteriorate if not frozen solid (Mollison, Mourant, and Race, 1948; Stirk, 1950), and it seemed likely that the tuberculous antibody behaved similarly. A further similarity found was that one sample of high titre Rhesus antiserum (saline anti-D agglutinin titre 128) showed no diminution in titre when tested by the standard method before and after heating at 56° C. for 30 minutes. It may be that the observed loss of titre of human sera on keeping is due to the action of a proteolytic enzyme active at about 4° C.

It must be admitted, however, that, when due allowance has been made for the technical difficulties enumerated above, the test from the clinical standpoint still leaves much to be desired. Two main problems arise: first, what significance should be attached to the existence in the sera of normal individuals of antibodies to an extract of tubercle bacilli; and secondly, why do the sera of about two-thirds of patients suffering from active tuberculosis fail to give significant titres with this test?

Tuberculosis differs from such diseases as syphilis, in which serological tests are of proved value. Relatively few people become syphilitic, and the finding of a positive reaction to a sero-diagnostic test is generally accepted as evidence of infection, but in tuberculosis most of the members of a community become infected, and, of these, the greater number rapidly overcome the infection. It is therefore not surprising to find tuberculous antibodies in low titre in the sera of normal individuals; in addition, such titres may reach higher levels after secondary subclinical stimulations. Thus, in the evaluation of this test, it is necessary to set an arbitrary dividing line based on a sufficient number of tests, titres below the chosen level occurring in healthy individuals, and those above this line being associated with clinical disease only. It thus appears that an arbitrary division might be set in this test at, or just below, a titre of 8; this is not, however, entirely satisfactory, in that only about one-third of the sera from tuberculous patients in our series were positive at this level. There are four possible explanations of this failure to obtain significant titres. The first is that the extract used for most of the tests is not sufficiently representative, and this aspect has been discussed above. On the limited evidence presented in Tables V and VI this does not appear a likely explanation, since the variations in titre amongst the different mammalian strains are not significant. A more decisive inquiry into this problem would consist of the isolation and extraction of organisms from "false negative" cases and the testing of such extracts with the patients' sera.

The second possible explanation for failure to detect antibodies is that, although they are being formed, there is liberation of an excess of bacillary products capable of reacting *in vivo* with the antibodies so that detection of the latter *in vitro* is precluded. This hypothesis of "swamping" might be investigated, as it is known that minute quantities of the sensitizing reagent in solution effectively block the reaction (Middlebrook and Dubos, 1948), and it would not be difficult to apply this method to "false negative" sera. Further consideration, however, suggests that the sensitizing factors might adhere to the patient's own red cells and thus be removed from the serum.

The third explanation for the failure is that antibodies were not present at the time of testing since they may not have been formed or may have been removed

y some mechanism other than that considered in the preceding paragraph. It is ell known that the maintenance of antibody levels in infectious disease is dependent ... continuous or repeated antigenic stimulation. For the purposes of this test it ems likely that the access of the breakdown products of tubercle bacilli to the ood stream is of greater importance than the nature and extent of the anatomical sion (Middlebrook, 1950). It must not be assumed, however, that the findings th this test are any indication of a patient's state of immunity, or even an indica- n that protective antibodies are present or absent. In the haemagglutination ction a deliberate selection of part of the breakdown products of tubercle bacilli s been made: a part which is capable of adhering to red cells. Protective anti- dies, if formed, may be directed against other bacterial constituents, or even ainst constituents elaborated in the lesion. The other aspect of this problem is t antibodies formed at an earlier date have disappeared at the time of the test. arly the liberation of antigenic material into the circulation ought to be greater m an early or extending lesion than from an older or quiescent one. If this is case, a close correlation between clinical activity and significant titres is to be ected. The following-up of individual patients should elucidate any variations itre associated with the progress of disease.

Yet another possibility has to be referred to: failure to detect "incomplete" a ibodies of the type encountered in Rhesus work (Wiener, 1944; Diamond and nton, 1945). The examination of a small number of "false negative" sera, d ted with 20% bovine albumin, was carried out and failed to reveal any agglutin ion; further investigation of this point is clearly needed.

In conclusion it may be said that a significant difference in antibody titres can b demonstrated between tuberculous patients and people without clinical tuber- c osis. The practical usefulness of the test is limited by the relatively small number of clear-cut reliable positive and the relatively large number of doubtful and "false negative" results. Improvements in technique and repeated examinations may well increase its value. High titres in doubtful clinical conditions where the diag- nosis lies between a tuberculous or another aetiology may be a most useful adjunct to diagnosis.

Summary

It has been shown that red cells can be sensitized with extracts of tubercle bacilli, becoming agglutinable by sera of tuberculous animals and patients. This principle has been used to try to obtain a test of clinical value.

Human Group O red cells were exposed to various extracts of tubercle bacilli, and the washed cells were tested using serial dilutions of human sera and Chown's capillary tube method of agglutination.

Unmodified "old tuberculin" was unsatisfactory as a sensitizing agent. Tuberculo-proteins do not appear to play a role in the reaction. A crude carbohydrate fraction was found to be the most suitable of the products tested.

Uninactivated sera and sera kept unfrozen for any length of time gave anomalous reactions. Fresh sera heated for 30 minutes at 56° C. and tested against red cells sensitized with a crude carbohydrate fraction obtained from the H 37 strain gave best results. Titres of 8 and over appear to be significantly associated with clinical

tuberculosis, but occurred in only one-third of the patients tested. Possible explanations for this are discussed, and suggestions are made for further investigations.

We wish to express our sincere thanks to Dr. H. H. Green, of the Veterinary Research Laboratories, Ministry of Agriculture and Fisheries, Weybridge, for providing valuable materials. Our thanks are also due to various friends who kindly provided sera of tuberculous patients.

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A SLIDE-CULTURE TECHNIQUE FOR DETERMINING STREPTOMYCIN SENSITIVITY OF *M. TUBERCULOSIS* IN SPUTUM

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It is now widely recognized that a rapid method for determining the streptomycin sensitivity of tubercle bacilli is highly advantageous in the management of tuberculosis undergoing, or about to undergo, treatment with this drug. Up to the present three different types of streptomycin sensitivity tests have been described. The first depends on preliminary isolation of the organism by culture on Lowenstein or similar media and the subsequent assay of the isolate in a fluid medium or by slide-culture (M.R.C. Report, *Lancet*, 1948 ; Giammalvo, Natsios, and Elton, 1949). This method is so slow (six to eight weeks) that its clinical usefulness is doubtful. In the second method the sputum is directly cultured after digestion on agar egg yolk media (Pyle, 1947 ; Karlson and Needham, 1948 ; Youmans, Ibrahim, Sweany, and Sweany, 1950), Lowenstein (Holt and Cruickshank, 1949 ; Hauduroy and Rosset, 1948) or potato-egg media (Anderson, 1949) containing appropriate dilutions of streptomycin. Even under the most advantageous conditions results are not obtained before the fourteenth day by this direct plating method. The third procedure is to use a slide-culture technique (Rubbo, Woodruff, and Morris, 1949 ; Sievers, 1949 ; Cummings and Drummond, 1949 ; Bernard and Kreis, 1949). The present communication is an extension of our previous work on slide-cultures and describes the development of a technique now being used as a routine test in our laboratories.

Methods

The essential problem in the direct micro-culture of tubercle bacilli is to achieve a simple method of treating the sputum without destroying its fixative properties or interfering with subsequent micro-growth. The choice of a fluid culture medium not affecting the activity of streptomycin but at the same time promoting rapid growth is also an obvious requirement. These points will be discussed.

Treatment of Sputum.—Sputa from four cases of pulmonary tuberculosis, two of which were receiving streptomycin therapy, were cultured on slides before and after treatment with 1% trypsin for 30 minutes, 5% oxalic acid for 30 minutes, 4% sodium hydroxide for 30 minutes, 6% (1.2 N) sulphuric acid for 20 minutes, and 23% trisodium phosphate for 24 hours at 37° C. After digestion the sputa were centrifuged at 3,000 r.p.m. for 30 minutes. The sediments were smeared on slides and allowed to dry in an incubator under cover of sterile petri dishes. The slides prepared from the tryptic digestion and the untreated sputa were sterilized by immersion in 6% sulphuric acid. All

slides were then immersed in a fluid serum synthetic medium and incubated for six days at 37° C. After incubation the slides were stained and examined microscopically for typical fasciculate growth (Fig. 1). In addition, all treated specimens were cultured on Lowenstein media for 30 days at 37° C.

FIG. 1.—Typical fasciculate. Micro-colonies of *Mycobacterium tuberculosis*. Medium: serum synthetic medium ρH 7.0–7.2. Incubation: six days at 37° C.

From the results shown in Table I the following conclusions were reached: (1) fasciculate growth was more pronounced in smears prepared from the untreated specimens; (2) digestion by NaOH and H_2SO_4 was unsatisfactory, as the smears tended to float off the slides during culture; (3) oxalic acid treatment inhibited growth on slides, but not on Lowenstein medium; (4) after trypsin or trisodium phosphate digestion growth was slight and variable although good growth was obtained on Lowenstein medium.

These preliminary results were confirmed by more extensive tests, particularly with trypsin and trisodium phosphate. In regard to tryptic digestion a fairly consistent pattern of results was obtained in which slide-culture was usually successful before streptomycin therapy was begun, but was seldom successful after eight days' treatment. This inhibi-

TABLE I

THE EFFECT OF DIGESTION OF SPUTA ON GROWTH BY SLIDE-CULTURE

Patient	Streptomycin Therapy	No Digestion Slide-culture	NaOH Digestion		H_2SO_4 Digestion		$Na_3PO_4H_2O$ Digestion		$(COOH)_2$ Digestion		Trypsin Digestion	
			Slide-culture	Lowenstein	Slide-culture	Lowenstein	Slide-culture	Lowenstein	Slide-culture	Lowenstein	Slide-culture	Lowenstein
H.H.	0	+++	±	++	+	++	±	++	−	++	±	++
M.M. ..	15 g.	++	±	++	±	++	±	++	−	++	±	++
P.P. ..	38.5 ,,	++	+	++	±	++	+	++	−	++	+	++
B.F.	60 ,,	++	+	++	±	++	±	++	−	++	−	++

TABLE II

THE EFFECT OF TREATMENT DESIGNED TO ELIMINATE CONTAMINANT ORGANISMS ON GROWTH BY SLIDE-CULTURE

Patient	Streptomycin Therapy	Degree of Growth Following									
		No Treatment		6% H_2SO_4 Treatment		CH_3OH Treatment		Penicillin and Sulphadiazine in Medium		CH_3OH Treatment and Penicillin and Sulpadiazine in Medium	
		Contaminants	T.B. Bacilli	Contaminants	T.B. Bacilli	Contaminants	T.B. Bacilli	Contaminants	T.B. Bacilli	Contaminants	T.B Bacilli
L.W.	Nil	+	−	−	+	±	±	−	+	−	+
M.W.	,,	+	−	−	+	+	−	−	+	−	+
B.C.	,,	±	−	±	+	+	±	−	±	−	±
M.M.	,,	+	−	−	+	+	−	+	−	+	+
R.T.	,,	+	±	−	+	+	−	−	±	−	+
H.H.	,,	+	−	−	++	+	+	+	+	−	+
K.T.	,,	+	−	−	+	+	+	−	+	−	+
F.D.	,,	+	−	−	+	+	−	+	−	+	±
T.N.	29 g.	+	−	−	++	+	−	−	+	−	+
P.P.	30 ,,	+	−	−	++	±	−	±	+	−	+
Comment on result		Unsatisfactory		Satisfactory		Unsatisfactory		Unsatisfactory		Unsatisfactory	

+++ signifies luxuriant growth. ++ signifies good growth. + signifies moderate growth. ± signifies slight growth. − signifies no growth. Serum synthetic medium *p*H 7.0–7.2. Incubation for six days at 37° C.

tion of growth occurred only with the slide-cultures, since the organisms were easily cultured from the same material on Lowenstein medium.

It was clear that the usual methods of treating sputa were of little value for the preparation of slide-cultures. From the practical point of view it is unlikely that this affects the sensitivity of the test, since Spendlove, Cummings, and Patnode (1949) have shown that concentration methods do not increase the number of detectable bacilli in a direct smear.

The problem of determining the simplest technique of destroying the varied population of non-acid-fast bacilli and yeasts present in sputum was studied. Four procedures were compared: (1) immersion of the smeared slide in 6% sulphuric acid for two minutes before culture in serum synthetic medium; (2) immersion of the smear in 70% methyl alcohol for five minutes; (3) immersion of the untreated smear in the medium to which 5 units per ml. of penicillin and 50 μg. per ml. of sulphadiazine had been added; (4) combination of (2) and (3). The treated slides in methods (1), (2), and (4) were washed in distilled water before immersion in the serum synthetic medium. The results for 10 sputa are listed in Table II.

It will be seen that the acid treatment gave the best results, in that fasciculate growth was observed in every specimen with slight contamination occurring only in one instance. Methyl alcohol treatment was invariably unsuccessful in controlling contaminant organisms whose growth tended to inhibit that of the tubercle bacilli. The penicillin-sulphadiazine combination was equally disappointing.

Further investigations of the acid treatment method showed that the number of contaminants decreased as the exposure time to acid increased but that this was accompanied by a decreasing size of fascicle formation. It was found that the optimal time of acid treatment was approximately four minutes, which is shorter than that recommended by other authors (Hauduroy and Rosset, 1948; Sievers, 1949; Cummings and Drummond, 1949; and Bernard and Kreis, 1949).

Choice of Fluid Medium.—Up to the present stage of the investigation we had used a serum synthetic medium described by Williston and Youmans (1949) for all cultural work. However, since Giammalvo *et al.* (1949) had used a saponated blood medium, Sievers (1949) a lysed defibrinated blood, and Bloch (1948) had indicated that chicken embryo extract was of value, we decided to modify the serum synthetic medium along the following lines.

Using the basic medium (SG) containing asparagin 0.5%, KH_2PO_4 0.5%, K_2SO_4 0.5%, glycerol 2.0%, magnesium citrate 0.15% in distilled water (*p*H 7), modifications were prepared by adding (*a*) 10% ox serum (SGS, serum synthetic medium as above), (*b*) 10% defibrinated blood (SGB), (*c*) 10% yeast extract (SGY), (*d*) 1% chick embryo extract (SGC), (*e*) 10% serum and 1% chick embryo extract (SGSC), and (*f*) 10% egg yolk (SGE). Also included in the series was the saponated blood medium.

Nine sputa from tuberculous patients were cultured on slides (after acid treatment) by immersion in these media for six days at 37° C. The results are summarized in Table III.

It will be seen that the improvement in the degree of growth obtained by supplementing the basic medium (SG) with serum (SGS) was equal to that effected by the various extracts. The addition of egg yolk and yeast extract caused films to deposit on the surface of the slides, making the microscopic observation of fasciculate growth difficult. Saponated blood medium prepared by lysing citrated blood with an equal volume of 1% saponin supported growth equally as well as did serum synthetic medium (SGS).

Examination of Slide-cultures.—Two methods of demonstrating tubercle bacilli and their fasciculate micro-colonies were open to us—either the Ziehl-Neelsen stain, using the

TABLE III

THE EFFECT OF MODIFICATION OF SERUM SYNTHETIC MEDIUM† ON GROWTH BY SLIDE-CULTURE

Medium	Code	Degree of Growth									Average Result	Comment
		Sputum										
		1	2	3	4	5	6	7	8	9		
Basic medium: (*a*) plus 10% serum ..	SGS	++	+++	+	++	+	++	+++	+++	++	++	Satisfactory
Basic medium: (*b*) plus 10% defibrinated blood	SGB	++	+++	±	+++	+	++	++	+++	+++	++	Satisfactory
Basic medium: (*c*) plus 10% yeast extract	SGY	++	−*	−*	++*	+	±*	+++	++	+	+	Unsatisfactory
Basic medium: (*d*) plus 1% chick embryo extract	SGC	++	−	−	−	−	−	−	−	−	−	Unsatisfactory
Basic medium: (*e*) plus 10% serum and 1% chick embryo extract	SGSC	+	+++	+	++	+	++	+++	+++	++	++	Satisfactory
Basic medium: (*f*) plus 10% egg yolk	SGE	++	±*	+	++*	±*	++	++*	++	++	+	Unsatisfactory
(*g*) Saponated blood medium		++	+++	+	++	+	++	+++	+++	++	++	Satisfactory

+++ signifies luxurant growth. ++ signifies good growth. + signifies moderate growth. ± signifies slight growth. − signifies no growth. * Film over slide making reading difficult. † See text for composition. Incubation for six days at 37° C.

light microscope, or the auramine stain (Hughes, 1943), using the fluorescent microscope. Since the latter is used as a routine method in these laboratories we adopted this procedure from the outset. Comparison, however, was made between two techniques with identical results, but the tediousness of the Ziehl-Neelsen method is so great, when compared to the fluorescent technique, that it is easy to appreciate the reluctance in accepting the slide-culture assay as a routine method. In view of the improvements and simplification of apparatus recently described by our colleague (Matthaei, 1950) for fluorescent microscopy we consider the classical method of Ziehl-Neelsen or its modifications (Hallberg, 1946) unnecessarily time-consuming and fatiguing, and do not recommend their use in assessing the results of slide-cultures.

Method.—The following method is now being used in our diagnostic laboratories for the rapid measurement of streptomycin or P.A.S. sensitivity of tubercle bacilli in sputum. Specially cut glass slides, 1 cm. by 6 cm., are prepared and sterilized by heat. Eight marked slides are mounted on metal slide carriers (Fig. 2) smeared liberally with sputum, choosing, where possible, caseous-like particles. The slides are dried in the incubator at 37° C. for 15 minutes and are then immersed in a petri dish containing 6% (1.2 N) sulphuric acid for four minutes. After this they are washed twice by immersion for two minutes in two petri dishes containing sterile distilled water. All these operations are carried out on a bench covered with brown paper, and, as a further precaution, it is useful to place the lid of the dish, which holds the acid or water, inside its cover as shown in Fig. 2.

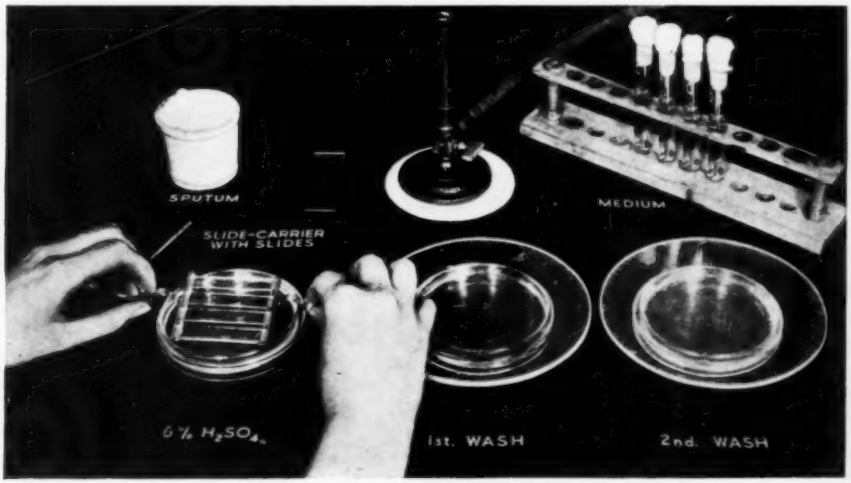

FIG. 2.—Slide-culture technique showing acid treatment of slides.

The treated slides are then transferred to eight small test-tubes, 10 cm. by 1.5 cm., holding 5 ml. of serum synthetic medium (SGS) or saponated blood medium. In preparing the former it is important to remove traces of soap from all glassware by washing first in dilute chromic acid cleaning mixture and then in water (six rinses). Each pair of tubes contains 0, 2, 10, and 100 μg. (microgram) per ml. of streptomycin. Although it has been our practice to add streptomycin immediately before a test, Youmans and Karlson (1947) have shown that the drug is quite stable at 10° C. in SGS medium

or at least 35 days. The tubes are sealed with paraffined plugs and incubated for six days at 37° C.

A slide from one of the control tubes is removed with forceps, fixed and sterilized by heating on a hot plate (90° C. for 15 to 30 minutes), and stained with warmed auramine-rhodamine stain for 10 minutes. It has been found that excessive heating of the slides before staining (viz., by passing the slide directly through the bunsen flame) results in a marked reduction in the number of stainable bacilli, and hence should be avoided. The stained smear is examined by fluorescent microscopy, using either ultraviolet light as a radiant or a 100-watt globe as described by Matthaei (1950).

Growth of the tubercle bacilli in the preparation can be readily detected by the presence of cord-like masses of golden acid-fast bacilli, best described as fasciculate micro-colonies (Fig. 1). If growth is observed on this slide the remainder are then examined. If, however, no growth is observed in the control, the cultures are incubated for a further four days. If fascicles do not form after 10 days' incubation it is unlikely that any growth will occur subsequently. On some occasions slight contamination of the medium has been noted, due particularly to yeasts, but this does not affect the titration except when the contamination is very heavy.

The end-point is the lowest concentration of streptomycin inhibiting fasciculate growth of the majority of acid-fast bacilli seen in the preparation. In some tests small fascicles when compared with the controls) and single cells are seen on the same slide indicating a mixture of partially resistant and sensitive (or non-viable) organisms. Such observations are of value in forecasting the development of resistance.

In view of the fact that the sensitivity of tubercle bacilli to streptomycin may be greatly influenced by the technique employed (Williston and Youmans, 1949) it was decided to investigate further the method just described. To this end the following tests were performed. Streptomycin-sensitive and resistant cultures of *Mycobacterium tuberculosis* H37Rv were prepared in Dubos and Davis liquid albumin medium (Dubos and Davis, 1946) containing 0.05% of Tween 80 to ensure a dispersed growth. The cultures were standardized to the same turbidity, then mixed in different proportions as shown in Table IV. To these mixed culture suspensions sterile ox serum was added to give a

TABLE IV

COMPARISON OF SLIDE-CULTURE ASSAY WITH TUBE DILUTION METHOD

Inoculum	Mixture	Slide-culture Assay (μg. streptomycin/ml.)			Tube Dilution Method (μg. streptomycin/ml.)		
		0	10	100	0	10	100
H37Rv sensitive H37Rv resistant	100% 0%	+++	−	−	+++	−	−
H37Rv sensitive H37Rv resistant	90% 10%	+++	+	±	+++	+	±
H37Rv sensitive H37Rv resistant	50% 50%	+++	++	++	+++	++	++
H37Rv sensitive H37Rv resistant	0% 100%	++	++	++	++	++	++

+++ signifies luxuriant growth. ++ signifies good growth. + signifies moderate growth. ± signifies slight growth. − signifies no growth. Serum synthetic medium *p*H 7.0–7.2. Incubation for six days at 37° C.

final concentration of 80% serum. This provided a bacterial suspension capable of adhering to the slides when cultured by immersion in fluid medium. The tests were made in duplicate from each suspension in serum synthetic medium containing 0, 10, and 100 μg. of streptomycin per ml. At the same time the suspensions were used to sow heavily 0.05 ml. inoculum and 5-ml. quantities of the same medium containing the same streptomycin dilutions. The slide-cultures and tube cultures were incubated at 37° C. and the results noted after six days' incubation.

It will be seen that the results obtained by the two methods were identical and that it was possible to detect resistant forms in a predominantly streptomycin-sensitive population (90%) by slide-culture. It was evident that slide-culture was as sensitive and gave the same results as the tube dilution method.

A second series of experiments was made with the object of discovering whether this direct method of assay yielded the same results as the indirect method. Tests were done with sputa of six patients by direct slide-culture and later with the same organisms isolated on Lowenstein medium. The latter were subcultured in liquid Tween albumin medium for six days to provide an inoculum for assay by the tube dilution method in serum synthetic medium. The results are shown in Table V, in which it will be noted that

TABLE V

COMPARISON OF SENSITIVITY DETERMINED BY DIRECT SLIDE-CULTURE AND TUBE DILUTION USING CULTURE ISOLATED FROM SPUTUM

Patient	Streptomycin Therapy	Lowest Concentration of Streptomycin Inhibiting Growth	
		Direct Slide-culture using Sputum	Tube Dilution using Isolated Pure Cultures
E.T.	Nil	2 μg. per ml.	2 μg. per ml.
L.M.	,,	2 ,, ,, ,,	2 ,, ,, ,,
N.P.	,,	2 ,, ,, ,,	2 ,, ,, ,,
E.D.	,,	2 ,, ,, ,,	2 ,, ,, ,,
B.F.	30 g.	>800 ,, ,, ,,	>800 ,, ,, ,,
P.P.	45 g.	>1,000 ,, ,, ,,	>1,000 ,, ,, ,,

Serum synthetic medium *p*H 7.0–7.2. Incubation for six days at 37° C.

no difference in sensitivity (or resistance) was observed between the organisms tested directly and those assayed indirectly from pure cultures.

It may be added that in both series the results were read after 6, 14, and 21 days' incubation without detecting any change in sensitivity. It appears that the short incubation time is equally as effective as longer periods of incubation.

Discussion

The emergence of streptomycin-resistant strains of tubercle bacilli *in vivo* is now well established. While the statistical evidence obtained by the Streptomycin Committee of the Veterans Administration (1948) indicated that resistant forms may develop in 40% of cases after 60 days' treatment the management of the individual case must be based on the clinical, radiological, and bacteriological evidence available. One important aspect of the latter is the determination of streptomycin resistance before and during therapy. As already pointed out, a number of direct and indirect methods have been described, but, in our opinion, slide-culture assay is the method of choice for routine purposes. Of the various techniques dealing with

slide-culture the method described here appears to satisfy the important clinical and laboratory criteria demanded of such a test. The procedure is technically simple, rapid in performance, and yields an accurate result six days after receipt of the specimen. Furthermore, extremely small quantities of sputa can be handled, and the medium is easily prepared and stored. Slide-cultures possess the advantages of direct plating, but without such disadvantages as homogenization of the sputa, the tedious preparation and storage of solid media, the delay in reaching a result (viz., not less than 14 days), and the confusion in readings due to contamination.

The present method differs from those previously described in the following details. (*a*) Direct smears are made from selected particles as recommended by Spendlove *et al.* (1949). (*b*) Slides are mounted on a slide carrier to reduce manipulation to a minimum (Fig. 2). (*c*) Sputum smears are sterilized by immersion in 6% H_2SO_4 for four minutes and then washed twice in distilled water, using a double-sided petri dish (Fig. 2). (*d*) Serum synthetic medium is used containing 0, 2, 10, and 100 μg. per ml. of streptomycin. Saponated blood medium would be equally satisfactory, but the Dubos and Davis Tween albumin medium is not recommended on the grounds that fasciculate or cord-like growth is inhibited in the presence of Tween 80 (Middlebrook, Dubos, and Pierce, 1947). (*e*) Growth is determined microscopically with the fluorescent microscope, using Hughes's stain (Hughes, 1943) and Matthaei's optical system (1920). These requirements simplify yet increase the reliability and safety of the test.

It is obvious that the test can only be applied to sputa in which organisms are visible by direct smear. This limitation is not so serious as it appears, since the most important cases requiring investigation are those in which the sputum is positive. The early exudative lesion found radiologically in a symptomless individual without sputum would not provide a suitable specimen for a streptomycin sensitivity either by slide or direct plate methods, but, should the lesion progress, then in all probability the sputum would become positive and hence suitable for slide-culture assay. Other pathological fluids containing visible tubercle bacilli can be treated by slide-culture, provided a suitable fixative, such as serum or albumin, is used.

So far over 100 sputa have been examined in these laboratories with consistently good results. Only on a few occasions has it been necessary to repeat the test because of technical imperfection of the method. As a result of this experience we arbitrarily designate grades of resistance as follows:

Sensitive strain	growth inhibited by 2 μg. per ml. of streptomycin.
Slightly resistant	growth in 2 μg. per ml. but inhibited in 10 μg. per ml.
Moderately resistant ..	growth in 10 μg. per ml. but inhibited in 100 μg. per ml.
Highly resistant	growth in 100 μg. per ml.

Summary

A description of a slide-culture method for determining streptomycin-sensitivity of tubercle bacilli in sputum has been presented. The method involves culture of the organisms as micro-colonies on the surface of glass slides immersed in serum synthetic medium containing three concentrations of streptomycin. As the method does not involve preliminary isolation of the tubercle bacilli a result can be given six days after receipt of the specimen. The method possesses the advantages of the direct plate without its practical disadvantages.

A good deal of comparative testing was undertaken with a view to assessing the reliability and accuracy of the slide-culture assay. It was found that the results were identical with those obtained in liquid medium and the method was capable of detecting resistant mutants in a predominantly sensitive population of cells.

Arguments have been advanced on the grounds of simplicity and rapidity that slide-culture is the method of choice for determining streptomycin-sensitivity of tubercle bacilli in sputum, provided the organisms are visible in the direct smear from selected particles.

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THE DETERMINATION OF THE SENSITIVITY OF *MYCO. TUBERCULOSIS* TO STREPTOMYCIN

BY

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Streptomycin is now widely used for the treatment of tuberculosis, and, as the development of resistance by tubercle bacilli is a common occurrence in certain forms of the disease, bacteriological control is important. There is consequently a definite need for a reliable and simple routine method of testing the sensitivity to streptomycin of the tubercle bacillus as the demand for the information is increasing steadily.

The method in general use requires the serial dilution of streptomycin in the fluid Tween-albumin medium, described by Dubos and Davis (1946) and in the M.R.C. report (*Lancet*, 1948). This has given consistent results, but the technique is complicated and time-consuming and there is considerable delay (six to eight weeks) in obtaining the results. Other fluid media, such as the synthetic medium of Proskauer and Beck (surface and deep), have not generally proved as satisfactory as the Dubos method although giving comparable results (Wolinsky and Steenken, 1947).

Various forms of solid media, in particular Herrold's medium, have been introduced, and it is claimed that they provide a rough estimate of the sensitive and resistant organisms in the inoculum; this information is not given by fluid media (Pyle, 1947; Karlson and Needham, 1948). Herrold's egg-yolk medium does not appear to have been widely used in this country. Holt and Cruickshank (1949) tried Lowenstein and Jensen's medium, containing only approximate amounts of streptomycin, and reported satisfactory results from the examination of sputum. Youmans, Ibrahim, Sweany, and Sweany (1950) carried out direct sensitivity tests on more than 2,000 sputum examples and considered that more accurate results were obtained with Herrold's medium than with inspissated media, in which the final concentration of streptomycin was only approximate owing to the heating of the medium after the addition of the antibiotic. Williston and Youmans (1949) tested a wide range of media and demonstrated that the type of medium had a definite influence on the sensitivity results; they considered that fluid media, in particular the original Dubos medium, had many disadvantages and recommended Herrold's medium for general use. The slide method of Pryce (1941) has been tried, but it seems to have distinct limitations as a routine test; the inoculum must contain large numbers of tubercle

bacilli and so direct tests with pathological material may give erratic results (Cummings and Drummond, 1949).

During the past three years sensitivity tests have been carried out on strains of *Myco. tuberculosis* isolated from tuberculous material, mainly cerebrospinal fluid, by means of the standard Dubos technique; trials with various solid media have also been made. The results are now reported.

Tween-albumin (Dubos) Medium

Technique.—The method recommended in the M.R.C. report (1948) has been closely followed; glucose and vegex were not added to the medium in which the final concentration of the bovine albumin was 0.3%. Primary isolation of the organisms was carried out either on the Lowenstein-Jensen medium or at times by guinea-pig inoculation, from which further cultures, and if necessary subcultures, were prepared in the Dubos medium in order to obtain a diffuse growth; the concentration of organisms was adjusted to an opacity roughly equivalent to tube No. 2 of the Brown opacity series and a 1/10 dilution was prepared for the assay. The standard strain, H37Rv, was used as the control in each series of tests. Readings were made after incubation at 37° C. for 10 and 14 days.

Results

Eighty-one strains of *Myco. tuberculosis* have been examined; these were isolated from 56 strains of tuberculosis—49 meningitis, five adenitis, and two pulmonary. Sixty-six strains were obtained from the cerebrospinal fluid and include 17 strains isolated at varying stages of streptomycin therapy, in some instances nine to 12 months after the beginning of the treatment; six strains were obtained at necropsy from lesions at the base of the brain (Table I).

TABLE I

SENSITIVITY OF *Myco. tuberculosis* TO STREPTOMYCIN (81 STRAINS)

Inhibited by ½ μg. or less	57
„ „ 1 μg.	22
„ „ 2 μg.	1*
Not inhibited by 600 μg.	1

* Necropsy strain

The results, in general, agree with those obtained by other workers (M.R.C. Report, 1948; Mitchison, 1949). Strains isolated before streptomycin therapy proved very sensitive, while the meningitis strains did not tend to develop resistance during treatment.

The resistant strain was isolated from a kidney removed at nephrectomy of a case of meningitis developing a renal lesion during the course of treatment; at the same time *Myco. tuberculosis* was also isolated from the cerebrospinal fluid, but this strain proved to be sensitive. It is therefore important to note that strains isolated from one particular focus of a widespread infection do not necessarily indicate the sensitivity of strains in the other lesions.

This method has given consistent results, but it has the great disadvantage of taking some six to eight weeks before the final result is available. It is usually necessary to institute treatment before the sensitivity results are available.

Solid Media

Two types of solid media have been tried: (1) fluid Dubos solidified by the addition of agar, and (2) Herrold's egg-yolk medium. These have been used in either petri dishes or screw-cap bottles as a slope.

Technique with Dubos Medium.—Agar (Davis) 1% was added to the fluid medium and 2.5-ml. amounts were distributed in bijou bottles before the addition of the bovine albumin. The medium was sterilized in the autoclave for 10 minutes at 10 lb. pressure. The melted medium was placed in the 56° C. bath and the sterile albumin solution added to each bottle together with the appropriate amount of the standard streptomycin solution, the final strengths of which were 0.25, 0.5, 1, 2, 5, and 10 μg. per ml. Petri dishes containing 15 ml. of the medium were also prepared using a similar technique.

Technique with Herrold's Medium.—The original formula was closely followed (Herrold, 1931a and b). This is:

Lab. Lemco	3 g.
Peptone (Evans)	10 g.
NaCl	5 g.
Agar (Davis)	10 g.
Water distilled	1,000 ml.

The *p*H is adjusted to 7.5, and 150-ml. amounts are distributed in 250-ml. flasks and sterilized by autoclaving for 15 minutes at 10 lb. pressure. Glycerin was not added.

The yolk of one fresh egg was added to each 150-ml. batch of medium at 56° C., making a final concentration of approximately 15% of egg yolk; the appropriate amounts of the standard streptomycin solution were carefully added at this stage; on cooling to 45° C. the medium was distributed in petri dishes or, as slopes, in bijou bottles (2.5 ml.).

In the meantime eggs, which should be fresh, were washed well first with soap and warm water and then with hot tap water, after which they were placed in methylated spirits for 15 minutes; they were then dried individually with a sterile cloth and placed in a sterile petri dish. A small opening was made at one end of the egg with sterile forceps and the white of the egg removed. The hole was next enlarged and the yolk poured into a sterile beaker, in which it was broken up with a sterile glass rod and then added to the agar base at 56° C.

The media were seeded and incubated at 37° C. for at least 14 days. This presented no difficulty in the case of the screw-cap bottles, but considerable care was necessary with the petri dishes in order to obtain satisfactory results. A convenient and simple procedure was to wrap each plate in paper and place in a covered glass jar.

Results

(A) **Solid Dubos.**—This proved a good medium for *Myco. tuberculosis*; growth on the streptomycin-free medium was apparent in some six days and became profuse in 10 to 14 days.

Parallel tests were made with the solid and fluid Dubos media using pure cultures of *Myco. tuberculosis*. The results given by the two types of media were in close agreement, i.e., with sensitive strains growth was inhibited by the presence of 1 μg. per ml. of streptomycin.

Attempts to obtain quick results were made by the direct seeding of the media with tuberculous material collected from patients. It is, however, essential that large numbers of tubercle bacilli should be present in the inoculum, and it is therefore not

surprising that cerebrospinal fluid proved unsatisfactory owing to the scanty numbers of bacilli present ; good results were, however, obtained with caseous material from human, as well as guinea-pig, sources by this technique.

In the case of cerebrospinal fluid, it was necessary first to isolate the tubercle bacilli on Lowenstein-Jensen medium and then to subculture in the Dubos fluid medium, which was suitable for inoculation when a general turbidity was apparent. The time saved in obtaining the final report of the sensitivity of a strain was therefore of little practical value.

(B) Herrold's Medium.—When freshly prepared, this proved an excellent medium for the tubercle bacillus ; obvious growth developed in three to five days and was luxuriant in seven to ten days.

Sensitivity tests were made mainly on pure cultures of tubercle bacilli, usually in the fluid Dubos medium, using (1) media in which varying amounts of streptomycin were incorporated, or (2) the filter-paper technique. Direct assays were made on a few samples of sputum from tuberculous patients.

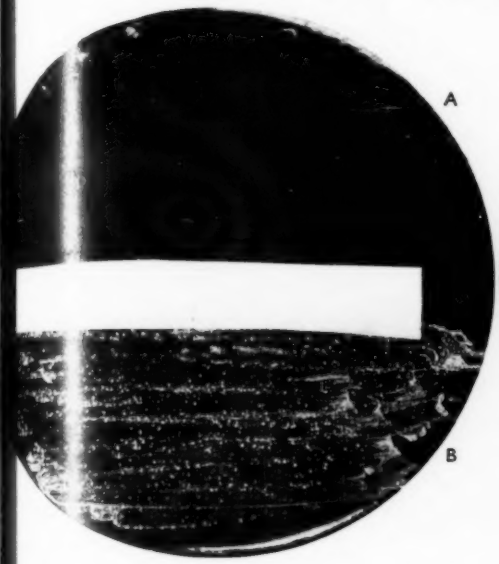
Streptomycin was incorporated in the medium to give a final concentration of 0.5, 1, 2, 5, and 10 μg. per ml. Comparative tests were made with the fluid and solid Dubos media. The results showed general agreement. It was, however, interesting to note that inhibition of growth was obtained by the presence of 1 μg./ml. in the solid Dubos medium, but in the case of the Herrold medium the addition of at least 2 μg./ml. was frequently required to produce this result. The influence of the medium on the sensitivity of tubercle bacilli has already been noted (Williston and Youmans, 1949). It is therefore important that, when the results of sensitivity tests are given in terms of units or μg., the medium used in the assay should be stated.

The preparation of the Herrold medium is relatively simple, and, when petri dishes are used, resistant colonies can be readily selected and, if necessary, several strains can be tested on one plate. It is also possible to obtain a quick result by direct plating when the inoculum contains many tubercle bacilli as in the case of many samples of sputum. These are distinct advantages over the fluid media.

The use of filter-paper strips or disks has proved useful as a crude and simple method of testing the sensitivity of organisms to the antibiotics, and it is widely used in routine practice. An attempt was therefore made to adapt this technique to the study of the tubercle bacillus. Disks were impregnated with varying concentrations of streptomycin—10, 20, 50, 100, 250, 500, and 1,000 μg./ml.—and then placed carefully on the Herrold media which had been heavily seeded with sensitive strains of *Myco. tuberculosis*. Incubation at 37° C. was carried out for two to three weeks. No effect was observed with strengths up to 100 μg./ml., but definite zones of inhibitions were produced by concentrations of 250 μg./ml. and greater.

A concentration of 500 μg./ml. was consequently adopted for the routine tests, in which strips of filter paper were mainly used. This proved a convenient method of testing several strains at the same time and obtaining a crude index of sensitivity of the organisms to streptomycin (Figs. 1 and 2).

Minor variations in sensitivity cannot, however, be detected by this simple technique. Cultures in a fluid medium are advisable, and there is therefore little, if any, saving of time, but, as several strains can be tested on one plate, it provides a relatively simple and economic technique.



1.—Streptomycin strip (1,000 μg./ml.) on Herrold's medium with (A) sensitive strain (H37Rv and (B) resistant strain RK (not inhibited by 600 μg./ml.). Fourteen days' incubation at 37° C.

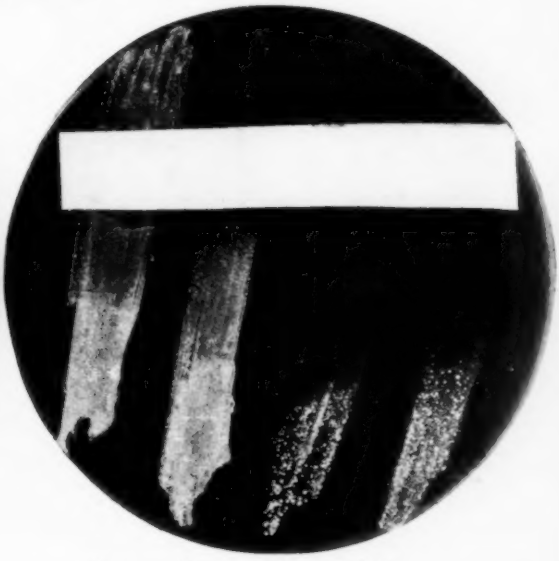


FIG. 2.—Streptomycin strip (500 μg./ml.) on Herrold's medium with (A) and (B) resistant strains (not inhibited by 60 μg./ml.) and (C) and (D) sensitive strains inhibited by 0.5 μg./ml. Fourteen days' incubation at 37° C.

Discussion

These results confirm the view that the fluid Dubos medium provides a consistent method for determining the sensitivity of *Myco. tuberculosis* to streptomycin. It is, however, tedious and time-consuming and has therefore a limited application in routine practice. Moreover, it does not provide any indication of the relative numbers of sensitive and resistant organisms, or enable the detection of a few resistant organisms in the inoculum. It does, however, give valuable information, provided the inoculum contains a great majority of either sensitive or resistant strains.

Solid media, in the form of Dubos or Herrold, have also given good results. Definite amounts of streptomycin can be added, and they provide a simpler and quicker method of making an assay, provided the inoculum contains large numbers of tubercle bacilli. These media have consequently proved very useful for the direct examination of such materials as sputum and urine, when a result may be obtained in seven to 14 days. They also provide a rapid method of detecting resistant strains.

When few bacilli are likely to be present in the original material, as in the case of cerebrospinal fluid, it is necessary to prepare the initial cultures on streptomycin-free media in order to obtain a suitable growth for the seeding of the assay media. In consequence the final results are not available for at least three to four weeks, instead of some six weeks with fluid Dubos. This difference is of little practical value, and it is unlikely to influence the initial course of therapy.

The relative simplicity of the solid media, particularly the strip technique, should prove useful as a screening test in routine laboratories. Strains giving irregular results by these tests could then be referred for a more complete assay to central laboratories, where the elaborate techniques are available.

Summary

The fluid Tween-albumin medium has given consistent results with strains of *Myco. tuberculosis* isolated mainly from cases of meningitis.

Solid media, in the form of Dubos or Herrold, have also given reliable results, but the time saved by their use in cases of tuberculous meningitis is of little practical value. They are particularly useful for the direct examination of material likely to contain large numbers of tubercle bacilli.

The filter-paper technique, using a streptomycin concentration of 500 μg. per ml., provides a simple, crude method of assaying sensitivity.

It is a pleasure to thank the Medical Research Council and Dr. J. Ungar for the supply of standard streptomycin and the Department of Clinical Photography for the photographs.

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THE PROTEOLYTIC ENZYME TEST FOR DETECTING INCOMPLETE ANTIBODIES

BY

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The specific agglutination by incomplete anti D antibodies of a saline suspension of red cells, after preliminary incubation with a solution of crystalline trypsin, has been previously reported (Morton and Pickles, 1947). During the past two years this method has been standardized and used as a test for the detection of incomplete antibodies. The trypsin-treated cells have been found to give reliable results and to be more sensitive than the concentrated albumin (Diamond and Denton, 1945) and anti-globulin (Coombs, Mourant, and Race, 1945) methods in the detection of weak incomplete anti D antibodies. The utilization of trypsin-treated red cells for the routine detection of anti Rh antibodies has been reported by Wheeler, Luhby, and Scholl (1950) and also in the studies of haemagglutinins in congenital and acquired haemolytic anaemias by Wright, Dodd, and Bouroncle (1949).

In view of the high cost of the crystalline trypsin previously used from the Plaut Research Laboratory, New Jersey, and of the unreliability of some of the commercial varieties of trypsin which have been tested, a search has been made for other proteolytic enzymes which would react in the same manner. This paper reports an investigation of proteolytic enzymes which could be used as a substitute for crystalline trypsin, the standardization of the test, and its reliability and specificity as a result of two years' experience in routine antibody determinations.

Proteolytic Enzymes as a Substitute for Trypsin

Crude filtrates of various organisms known to have proteolytic activity have been tested for their ability to imitate the action of trypsin on red cells.

The following organisms were grown in broth culture and on 0.5% heart agar and the filtrates examined at intervals from two to 14 days.

> *B. subtilis*; *B. cereus*; *B. circulans*; *B. licheniformis*; *Proteus*; *Ps. pyocyanea*; *Actinomyces* A 13; *Actinomyces* Polychrom NCTC 4599; *Actinomyces* Ruber; *Streptomyces sp.* (Dunn School of Pathology, Oxford); *Streptomyces albus* (Maxted, 1948).

The only organisms found to show specific effects were the two strains of *Streptomyces albus*.

In addition, commercial preparations of papain, pepsin, and trypsin were tested, and crystalline trypsin (Armour) and B.D.H. trypsin commercial and "refined" were found to give reliable results. The glycerol in solutions of trypsin such as liquor trypsini co. (Allen and Hanburys) often caused haemolysis of the red cells in the effective range of enzyme concentration. Papain solutions 1% and 10% were found to be suitable, but in view of the difficulty of standardizing the E^H were not intensively investigated. The pepsin solution was found to be inactive.

Preparation of Stock Enzyme Solutions

Commercial or refined trypsin powder (B.D.H.) was mixed with 0.05 N HCl in the proportions of 200 mg. to 1 ml., shaken at intervals, and left overnight at 4° C. The following day the supernatant fluid was removed after spinning down the undissolved particles and the solution kept at 4° C. Crystalline trypsin (Plaut Research Co., New Jersey, and Armour Laboratories) containing 50% $MgSO_4$ was made up as a solution of 10 mg./ml. in 0.05 N HCl. These acid solutions have shown no diminution in activity over a period of four months. Immediately before use the stock enzyme preparations were diluted 1 : 10 (v/v) with 0.1 M phosphate buffer *p*H 7.7.

Streptomyces albus spores were sown on a 1.25% agar medium in Roux bottles as described by Maxted (1948). After four to six days at 30–37° C., when good sporulation is usually observed, the bottles were placed at −20° C. for 12 hours and then allowed to thaw out, and the fluid which separated from the agar was passed through a Seitz filter. Stock filtrates were kept frozen at −20° C., but for daily use they were kept sterile by the addition of an equal volume of 0.1% (v/v) sodium azide and kept at 4° C. when not in use. A comparison was made of the activities of the stock preparations, unbuffered and buffered to *p*H 7.5, which had been kept at −20° C. continuously, and the filtrate after the addition of sodium azide, which had been kept on the bench daily and at 4° C. overnight. After a period of three months no differences could be found in the ability of these various solutions to change red cells.

Besides the crude culture filtrates of the strain of streptomyces a partially purified preparation was made available by Dr. van Heyningen and Dr. Born. The organism had been grown in deep aerated culture ; the enzyme material in solution was concentrated by evaporation of the fluid under reduced pressure and precipitation by saturation with ammonium sulphate and dried. The dried material, which has been termed " streptomyces lysin," was dissolved in 66% glycerol, and the stock solution was prepared at a concentration of 2 mg./ml. The glycerol solution was stored at 4° C. and showed no deterioration over a period of six months. For use the glycerol solution was diluted at least 1 : 40 (v/v) with 0.1 M phosphate buffer. With higher concentrations of the glycerol, haemolysis of the red cells occurs on washing the cells after treatment. The crude solutions were diluted with 4 to 9 volumes of buffer before use, depending on the activity.

Preparation of Enzyme-treated Red Cells

For brevity the preparation of the enzyme-treated red cell will be referred to as the P.E. (proteolytic enzyme) test or method subsequently in this paper.

Group O blood of suitable Rh type to include positive and negative controls for most of the Rh antigens (R_1R_1 CDe/CDe ; R_2r cDE/cde ; rr cde/cde) is taken into sodium citrate or citrate saline (0.5% sodium citrate in 0.85% sodium chloride), and washed three times with about 20 volumes of normal saline to remove the serum trypsin inhibitor. To one volume of packed washed cells 4 volumes of diluted enzyme in 0.1 M phosphate buffer *p*H 7.7 is added. The following enzyme solutions have been used, and the figures refer to the final concentrations in buffer as determined after standardization of the test.

Crystalline trypsin.	Plaut	1.0 mg./ml.
„ „	Armour	1.0 mg./ml.
B.D.H. commercial	.. ..	20 mg./ml.
„ refined	.. ..	8–20 mg./ml.
Crude actinomyces filtrate	.. ..	1/5–1/10 (v/v) dilution of crude filtrate
Streptomyces lysin	.. ..	0.05 mg./ml.

The mixture is gently shaken and placed in a water bath for 30 minutes at 37° C. The cells are then washed once and made up to a 5% (v/v) concentration in saline. These manipulations can be simply performed in graduated centrifuge tubes. The cells retain their new properties for at least 24 hours if prepared with sterile precautions and kept at 4° C. Cells prepared in bulk and stored at 4° C. as packed cells have remained active for four days.

Technique of P.E. Test.—In testing sera for the presence of the anti D antibody, 0.1 ml. or one large drop of the serum is pipetted into each of three small tubes (5 × 0.5 cm. is a convenient size) and placed in a water bath at 37° C. for a few minutes to equilibrate, and an equal volume of the warmed cell suspension of each type of cell is then added. The cell-serum mixture is shaken and allowed to sediment. The agglutination given by many incomplete antibodies will show within a few minutes of mixing, but we have found that the sediment pattern obtained after incubation for one half to one hour gives the most easily read results, as during the sedimentation a ragged edge is often seen. The concentration of the red cells is important, as too light a suspension may fail to give a clear-cut sediment pattern. After this time the majority of antibodies show the agglutinated cells to have formed a solid button, which by gently rotating the tube may be dislodged from the bottom of the tube, whereas the negative cells flow evenly down the side of the tube when it is tilted. The macroscopic reading is usually sufficient if negative control cells and a weak incomplete anti D antibody are included in the test, for the agglutination is firm and not as easily dispersed by shaking as are the agglutinates obtained with saline agglutinins and the anti-globulin test.

A non-specific agglutination is shown by many sera with P.E. cells at 20° C., but by previously warming the cells and serum and by incubating the mixture for one half to one hour this can be abolished. In most cases it appears to be due to the enhancement of non-specific cold agglutinins, but a few sera have been encountered which give a slight panagglutination just visible macroscopically at 37° C. with all cell suspensions, and has been more often observed with cells which have been treated with trypsin than with streptomyces lysin. In most cases it indicates over-treatment of the cells with enzyme, and precautions against its occurrence are discussed under standardization of the test.

Standardization of the Test.—The changes in the red cells after treatment by proteolytic enzymes can be measured by the degree of agglutination and end-point titre obtained on titration of a standard incomplete antibody. Variations in cell and enzyme concentration, *p*H, and time of incubation result in differences in the sensitivity of the test as judged by the figures given on titration; accordingly the maximum titre with a standard incomplete antibody, with no panagglutination of the cells with normal serum, has been deemed to represent the full change in the cells and has been used as a standard.

Two incomplete anti D sera (titre in albumin 1 : 8–16) were selected for the standardization tests and kept at −20° C., and in these experiments serial dilutions of the sera were made in 1-ml. volumes and the various serum dilutions transferred by standard droppers to sets of small tubes. One volume of packed, three times washed, cells and four volumes of enzyme solution, giving a cell concentration of 20%, was chosen arbitrarily as being easy to work with, and both the concentrations of the enzymes and the time of incubation with the red cells were varied. Since normal serum contains a potent inhibitor of proteolytic enzymes the reactions could be stopped at any particular time by the addition of an excess of normal serum before washing or by rapidly spinning down and washing the red cells three times with saline. The cells after treatment were made up to a 5% concentration in saline, and the amount of change produced in the red cells was measured by titrating the cells against a standard incomplete serum.

The majority of the standardization experiments were performed with one batch of streptomyces lysin and crystalline trypsin (Plaut Research Laboratory). The concentrations of the other enzyme preparations were adjusted to give equivalent results, and under the standard conditions 1 mg./ml. of crystalline trypsin (Plaut Research Co.) was equivalent to 1 mg./ml. crystalline trypsin (Armour Laboratories), 8 and 20 mg./ml. refined B.D.H. trypsin (two batches), 20 mg./ml. B.D.H. trypsin, and 0.05 mg./ml. streptomyces lysin.

TABLE I

EFFECT ON THE ANTIBODY TITRE OF INCREASING THE ENZYME CONCENTRATION AND INCUBATION TIME WITH RH-POSITIVE CELLS

Concentration of Streptomyces Lysin (mg./ml.)	Incubation Time with Cells	Dilution of Incomplete Anti D Antibody					
		1/10	1/20	1/40	1/80	1/160	1/320
0.025	0	1	—	—	—	—	—
	15′	C	C	3	1	—	—
	30′	C	C	4	2	—	—
	60′	C	C	4	3	1	—
0.05	0	C	2	—	—	—	—
	15′	C	C	4	3	—	—
	30′	C	C	4	3	2	—
	60′	C	C	C	3	2	—
0.1	0	C	C	2	—	—	—
	15′	C	C	C	3	2	—
	30′	C	C	C	3	2	—
	60′	C	C	C	3	2	—
Buffer control ..	60′	—	—	—	—	—	—

C = single clump of cells; 4 = 3–4 large agglutinates; 3 = several agglutinates; 2 = small agglutinates visible macroscopically; 1 = a few very small agglutinates.

Results

Table I shows the results obtained on titration of red cells exposed to increasing concentrations of streptomyces lysin at 15-minute intervals up to one hour. With a concentration of enzyme of 0.1 mg./ml. a maximum titre was obtained within 15 minutes, and no change in the pattern or type of agglutination was noted by prolonging incubation for a further 45 minutes. The enzyme used at half this concentration gave the same reading after 30 and 60 minutes, and with the enzyme used at a concentration of 0.025 mg./ml. this titre was barely reached within one hour.

With weaker concentrations of enzyme full activity of the cells could not be obtained even by prolonging the period of incubation up to eight hours, as shown in Table II. This is presumably due to the degradation of enzyme at 37° C. at *p*H 7.7, and we have found that after eight hours the activity of the trypsin as measured by its action on casein was reduced by 50%.

Cells exposed either to trypsin or streptomyces lysin develop non-specific agglutination with normal sera if the concentration of the enzyme or the time of incubation is excessive. This panagglutination occurs more rapidly with higher concentrations of enzyme and varies with different sera. Many sera never show non-specific agglutination, but a few show panagglutination with P.E. cells at the stage of development of the maximum titre as may be seen in Table III, which shows the effect of excessive concentration and time. The sera numbered 3 and 7 were both from

TABLE II

FAILURE OF WEAK ENZYME SOLUTIONS TO GIVE A MAXIMUM TITRE OF THE STANDARD ANTIBODY

Concentration of Enzyme (mg./ml.)	Incubation Time with Cells	Titration of Incomplete Anti D Antibody						Normal Serum
		1/10	1/20	1/40	1/80	1/160	1/320	
rypsin, 0.25	30′	C	C	2	—	—	—	—
	2 hr.	C	C	2	—	—	—	—
	4 ,,	C	C	2	2	—	—	1
	8 ,,	C	C	2	2	?	?	2
rypsin, 1.0	30′	C	C	C	4	1	—	—
treptomyces lysin, 0.05	30′	C	C	C	3	2	—	—

TABLE III

NON-SPECIFIC REACTION OBTAINED WITH EXCESSIVE CONCENTRATION OF ENZYME (CRYSTALLINE TRYPSIN) OR TIME OF INCUBATION WITH RED CELLS

ncubation Time (Hours)	Concentration of Enzyme (mg./ml.)	Non-antibody-containing Sera												Saline	Titre Against Standard Anti D
		1	2	3	4	5	6	7	8	9	10	11	12		
2	2	1	—	2	—	1	1	2	—	—	—	—	—	—	1/320
	1	1	—	2	—	1	—	2	—	—	—	—	—	—	1/320
	0.5	1	—	1	—	—	—	2	—	—	—	—	—	—	1/160
1	2	—	—	1	—	1	1	2	—	—	—	—	—	—	1/320
	1	—	—	1	—	—	—	2	—	—	—	—	—	—	1/320
	0.5	—	—	1	—	—	—	1	—	—	—	—	—	—	1/160
½	2	—	—	1	—	1	—	1	—	—	—	—	—	—	1/320
	*1	—	—	—	—	—	—	1	—	—	—	—	—	—	1/320
	0.5	—	—	—	—	—	—	—	—	—	—	—	—	—	1/160
¼	2	—	—	—	—	—	—	1	—	—	—	—	—	—	1/320
	1	—	—	—	—	—	—	1	—	—	—	—	—	—	1/80
	0.5	—	—	—	—	—	—	—	—	—	—	—	—	—	1/40

* The normal time of incubation and concentration of trypsin is set in a panel.

individuals with severe staphylococcal infections, and it may be that their sera contained a non-specific factor causing a type of pseudo-agglutination. From the point of view of detection of specific antibody these sera react with the patient's cells in the same manner as with all other cells, and the factor causing agglutination is not absorbable with P.E. cells and is abolished by dilution of the serum with an equal volume of saline. In this way it is analogous to the pseudo-agglutination due to rouleaux formation, which is often seen in sera from patients with high sedimentation rates, though this type of pseudo-agglutination is much reduced with P.E. cells and has rarely been observed.

The effect of various diluents on the panagglutination was tried by washing and resuspending the cells after excessive treatment with trypsin, with normal saline, 0.5% sodium citrate in 0.85% saline, 3.8% citrate, 0.1 M phosphate buffer and Ca borate buffer *p*H 7.2. No differences were observed with the panagglutinating sera

or the normal sera with the various diluents, and the titre against the anti D antibody remained the same, though with 3.8% sodium citrate the agglutination with this antibody was more easily broken up.

One serum has been encountered which has given a strong positive agglutination reaction with streptomyces lysin-treated cells, to a titre of 1 : 8, but all other antibody tests, including the trypsin test, were negative. The antibody was absorbable by streptomyces lysin-treated cells though not by trypsin-treated cells or by normal cells, and appeared to be a specific antibody analogous to that produced by the immunization of rabbits with streptomyces lysin-treated cells. Such sera, though rare, need to be critically analysed as they may simulate the results obtained with the sera of acquired haemolytic anaemias (Wright *et al.*, 1949). In this case there was no evidence of increased haemolysis (Hb 15 g./100 ml., reticulocytes 0.6%, direct Coombs test negative, cold agglutinins titre 1 : 2).

The standardization of the various brands of trypsin by their caseinolytic activity (Kunitz, 1947) has been attempted, and it was found that the caseinolytic activities did not bear any constant relationship to the activity in the production of P.E. cells. Table IV shows the degree of agglutination obtained with an incomplete anti D

TABLE IV

SENSITIVITY OF P.E. CELLS* INCUBATED WITH VARIOUS BRANDS OF TRYPSIN AT EQUIVALENT CASEINOLYTIC ACTIVITIES WITH AN INCOMPLETE ANTI D ANTIBODY

Trypsins	Concentration (mg./ml.)	Dilutions of Incomplete Anti D Antibody					
		1/10	1/20	1/40	1/80	1/160	1/320
Plaut	1.0	C	C	C	3	2	—
Armour	0.48	C	C	C	2	—	—
B.D.H. refined ..	6.56	C	C	C	3	2	—
,, ,, ..	7.67	C	C	C	3	1	—
,, crude ..	5.79	C	4	3	—	—	—

* Cells sensitized with 4 volumes of enzyme for 30 minutes.

antibody with the various types of trypsin at equivalent caseinolytic activities. The effective range of concentration of trypsin for the production of P.E. cells appears to be very high as the concentration of enzyme necessary for the trypsinization of the cells was 40 times that used for the determination of the caseinolytic activities. This may be due to the fact that the surface of the red cell is a living membrane and therefore relatively insusceptible to proteolysis, or may be explicable as the action of an unidentified enzyme present as a contaminant in trypsin and varying in concentration during refinement and crystallization.

Tests with Antibodies Detected with the Albumin and Coombs Tests

Eighty-six sera known to contain incomplete anti D antibodies, which had been stored for one to three years at −20° C., were examined using the P.E. method in parallel with the albumin test (using 25% human albumin) and the antiglobulin (Coombs) test. With the P.E. method all these sera with the Rh-positive cells gave a 4+ reaction, and in most the cells formed a solid clump, whereas the Rh-negative control cells were completely negative ; among them were three sera that had given

ozones with albumin and several which had given negative reactions with the oombs test. The results indicated that the P.E. test was the more sensitive method r the detection of antibodies, and this has been confirmed by the higher titration lues obtained by the P.E. method and also by the results obtained with the test unselected sera sent for antibody determinations.

During the past two years 1,176 sera have been examined by the P.E. test in rallel with the routine methods with saline, 25% human albumin, and anti-globulin sts. The majority of the sera were from Rh-negative antenatal patients, though out 300 were from a series of post-transfusion cases whose sera were being amined for the development of specific antibodies. Sixty-four of these sera were und to contain anti D antibodies, and the results are shown in Table V.

TABLE V

ANALYSIS OF THE RESULTS OF ANTIBODY TESTS ON 64 SERA

	No. Positive	Albumin		Coombs		Proteolytic Enzyme	
		+	−ve	+	−ve	+	−ve
line agglutinating antibodies —not enhanced	2	2	0	2	0	2	0
eak agglutination with saline; stronger with tests for incomplete antibodies	8	8	0	6	2?	8	0
complete antibodies	54	49	5	36	18	54	0

In the sera of several of the antenatal patients the P.E. test gave the only positive result on the first testing, but as the pregnancy proceeded subsequent observations showed all the tests for incomplete antibodies becoming positive. The following cases illustrate this.

Mrs. C. F.—Para. 1. First child normal. Tested when three months pregnant. P.E. test ++. All other tests negative. Tested when eight months pregnant. P.E. test ++. Other tests weakly positive. Tested when eight and a half months pregnant. Incomplete anti D antibody (titre 1 : 1 albumin). Cord blood of infant was group R_1r; Coombs test positive. Mildly affected infant, who recovered with one transfusion.

Mrs. E. N.—Para. 2. Both children normal. Tested when six months pregnant. P.E. test ++++. Albumin ?+. Indirect Coombs test negative. Tested when eight months pregnant. P.E. test ++++. Incomplete anti D (titre 1 : 4 albumin). Cord blood of infant was group R_1r; Coombs test positive. The infant was transfused at 14 days because of the development of neonatal anaemia.

Mrs. H.—In 1947 the third child died with icterus gravis aged 4 days. Maternal antibodies agglutinated anti D 1 : 4. On October 17, 1949, Mrs. H. was admitted with a miscarriage at three months. Antibody tests: saline 0; albumin +; Coombs test ?+; P.E. test ++++.

The ease of interpretation and the clear-cut results obtained when the results with other tests are equivocal have led us to use the P.E. test as the final arbiter as to whether anti D antibodies are present or not. With some batches of albumin the tendency for rouleaux formation often makes the differentiation from true agglutination difficult to interpret, and with weak antibodies the antiglobulin test very often fails to show agglutination.

Titrations.—Agglutinating anti D antibodies which show no enhancement of titre by the Coombs technique and a lowered titre in albumin have not shown more than one serial dilution difference in titre with P.E.-treated cells, though the agglutination at the various dilutions was much firmer than with normal cells. The example illustrated in Table VI was from a woman in whom an antibody test was negative at

TABLE VI

THE REACTIONS OF AN AGGLUTINATING ANTI D SERUM WITH NORMAL AND P.E.-TREATED CELLS

	Mrs. S. Serum at Delivery							
	Dilution of Antibody							
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Saline	4	4	3	3	3	2	1	—
P.E. cells	C	C	C	C	4	3	2	1

seven and a half months' gestation but who showed an agglutinating anti D antibody for one month before delivery; the cord blood sample gave a very weak positive direct Coombs test, and the infant remained clinically normal.

With sera containing incomplete antibodies, however, the P.E.-treated cells have shown higher titration values than any other method. The average increase in titre being about four to 20 times above the Coombs or albumin titration values, Table VII

TABLE VII

THE TITRES OBTAINED WITH THE VARIOUS TESTS FOR SIX ANTI D SERA

	Tu	Di	Bi	Bu	Ba	Mo
Saline	0	1/2	0	0	1/4	0
"Conglutination" (Wiener, 1945) ..	1/32	1/32	1/8	1/2	1/16	1/4
Dilutions in serum, cell suspended in 25% albumin	1/64	1/16	1/16	1/2	1/16	1/4
Dilutions and cells in albumin (Diamond and Denton, 1945)	1/16	1/4	1/8	1/4	1/8	1/4
3 parts serum 1 part albumin (Wiener, Hurst, and Sonn-Gordon, 1947) ..	1/128	1/32	1/64	1/8	1/64	1/4
Antiglobulin test (Coombs, Mourant, and Race, 1945)	1/32	1/8	1/4	1/8	1/32	1/8
Trypsin	1/256	1/128	1/64	1/32	1/128	1/16

sets out the final titres of four incomplete sera and two sera containing both agglutinating and incomplete antibodies examined by five methods for incomplete antibodies. These six sera were all tested within three weeks of collecting the samples. As shown in Tables I and VI the end-points of the titrations with P.E. cells is very sharp, and the change from a 4+ or complete agglutination to no reaction covers only two or three tubes in the serial dilution.

Other Rh Antibodies.—Incomplete antibodies of specificities anti C, E, c, e, and anti C^w have shown agglutination with the P.E. test and except for the anti C^w

rum have shown much the same order of increased titration values as was seen with the incomplete anti D antibodies. An incomplete anti E antibody which

TABLE VIII

ANTIBODIES OF SPECIFICITY ANTI C, E, c, e, C^w

Serum	Cell Type	Titres Given with Different Techniques				
		Saline	Albumin	Coombs	P.E. Test	
					Trypsin	Streptomyces Lysin
Anti E	R_2r	0	1:2	1:2	1:8	1:8
*(No)	R_1R_1	0	0	0	0	0
Anti E	R_2r	1:4	1:8	1:32	1:256	1:128
	R_1R_1	0	0	0	0	0
Anti C	R_1R_1	0	1:4	1:2	1:64	1:32
*(1)	R_2r	0	0	0	0	0
Anti c	R_2r	0	1:4	1:8	1:64	1:64
C	R_1R_1	0	0	0	0	0
Anti e	R_2r	0	1:4	1:2	1:8	1:8
*(1)	R_1R_1	0	1:16	1:32	1:256	1:256
Anti C^w	C^w De/cde	0	1:2	1:2		1:2
*(No)	cde/cde	0	0	0	0	0

* Sera given by Dr. Race.

developed after transfusion was first identified by a positive P.E. test and later became positive with the albumin test. The lack of increased titre of the anti C^w serum may be explained by long storage or may be a fundamental lack of reaction of the antibody with P.E. cells as has been seen with incomplete antibodies of other specificities. In view of the infrequent occurrence of the anti C, E, c, e, antibodies and their value in genotyping, it seems that it would be possible to utilize the P.E. method in genotyping with incomplete antibodies giving low titres by the other methods.

Antibodies of Other Specificities.—Investigations with the P.E. method on agglutinating and incomplete antibodies unrelated to the Rh system have shown various types of reaction. In the systems involving anti A, anti B, anti Lu^a, anti Le^a, and anti P agglutinating antibodies no significant differences were obtained in the titre though there was more intense agglutination with P.E. cells than with normal cells. An incomplete anti Le^a serum showed no reaction.

With agglutinating sera of specificity anti M, anti N, and anti S, cells treated with crystalline trypsin (Plaut Research Co.) showed a slight increase of titre and avidity of reaction, but with all other brands of trypsin and also with the proteolytic enzyme from *Streptomyces albus* there was destruction of the antigen locus, the red cells after treatment giving no agglutination and failing to absorb specific antibody. The rate of destruction of these loci varied with the various enzyme preparations and was most rapid with streptomyces lysin. An incomplete anti S gave agglutination with trypsin-treated cells. One example each of an incomplete anti Fy and anti Kell showed no agglutination with either trypsin or streptomyces lysin-treated cells.

The non-specific incomplete antibody found in cases of acquired haemolytic anaemia has been found to give agglutination with P.E. cells, but as an antibody reacting with cells treated with streptomyces lysin has been observed in an apparently normal individual, it would appear that a strict evaluation of its significance should be made in any individual in which such a reaction is found.

These findings are summarized in Table IX and will be discussed more fully in a subsequent paper on the effect of proteolytic enzymes on the surface of the red

TABLE IX

THE PROTEOLYTIC ENZYME TEST WITH SERA OF VARIOUS SPECIFICITIES

Specificity	Type of Serum	Trypsin (Plaut)	Streptomyces Lysin
Anti A }	Non-immune, agglutinating	Slight increase in titre and avidity	Slight increase in titre and avidity
,, B }	,, ,, ,,	,, ,,	,, ,,
Anti P	,, ,, ,,	,, ,,	,, ,,
Anti M }	,, ,, ,,	,, ,,	No reaction
,, N }	,, ,, ,,	,, ,,	,, ,,
Anti S	? Immune, agglutinating	,, ,,	,, ,,
	? ,, incomplete	,, ,,	,, ,,
Anti Le^a	? ,, agglutinating	,, ,,	Slight increase in titre and avidity
	? ,, incomplete	Haemolysis	No reaction
Anti Lu^a*	,, agglutinating	No change	No change
Anti Kell*	,, incomplete	No reaction	No reaction
Anti Fy†	,, ,,	,, ,,	,, ,,
Immune antibody found in haemolytic anaemia with positive Coombs test ..	,, ,,	Agglutination	Agglutination

* Serum given by Dr. Mourant. † Serum given by Dr. Mollison.

cell. Except for the immune antibody in cases of acquired haemolytic anaemia only one serum of each type has been tested, and they were all of human origin.

Discussion

The recognition of the importance of the incomplete form of the anti D antibody has led to the development of numerous techniques for its identification. Many are not sufficiently sensitive for routine work, and others are cumbersome or impractical where large numbers of tests, such as those connected with antenatal work, have to be carried out. The P.E. test described here has received an extensive trial over the past two years and has shown many advantages in routine work. Rouleaux formation, which is difficult to overcome with any of the methods in which enhancement of agglutination is obtained with serum, plasma, or albumin, is largely abolished, and the Coombs test is apt to be laborious for routine work

because of the washings involved. The preparation of the cells by enzyme treatment can be performed in bulk, and the cells are stable for at least 24 hours if kept sterile at 4° C., thus allowing emergency work to be accomplished very easily and rapidly. The results obtained have shown that the test is more sensitive than the albumin test and that once an enzyme preparation has been standardized the reagents remain stable for several months. Although crude enzyme preparations can be used, it would seem desirable to use the more refined products, and especially valuable would be a supply of refined streptomyces lysin, which in our hands has given much less non-specific reactions combined with more rapid activity than crystalline trypsin. The differences observed with various brands of trypsin and streptomyces lysin with sera of various specificities have shown that the presence of contaminating proteolytic enzymes which easily destroy some blood groups of the red cells make the test unreliable as a routine method for all types of antibody determinations, but for the Rh group it has never failed to show agglutination with incomplete antibodies.

Summary

The technique and standardization of a method for the detection of Rh antibodies using P.E.-treated red cells have been described, together with the results of its use in routine tests for immune iso-agglutinins. The test has been found to enhance agglutination and diminish rouleaux formation with all types of incomplete anti Rh antibodies, and to be the most sensitive method for their detection. The pattern of agglutination with saline-agglutinating sera of specificity anti D, anti A, anti B, anti Le^a, anti Lu^a, and anti P is not altered when using enzyme-treated cells, but the agglutination with anti M, anti N, and anti S anti sera may be completely abolished with some brands of trypsin, and agglutination cannot be obtained with incomplete antibodies of specificity anti Kell and anti Fy.

We are greatly indebted to Dr. van Heyningen and Dr. Born for much help and advice and most generous gifts of streptomyces lysin; to Dr. Race for samples of incomplete sera of specificity anti C, anti C^w, anti e, and anti E, to Dr. Mourant for anti Kell and anti Lu^a, and Dr. Mollison for anti Fy.

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THE EFFECT OF A DIRECT ELECTRIC CURRENT ON NORMAL AND ANTIBODY-SENSITIZED RED CELLS

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During experiments on the effect of direct electric currents on red cells suspended in saline an unexpected phenomenon was observed. Normal red cells showed agglutination and lysis, but if the red cells had been sensitized by antibodies there was a marked difference in the degree of agglutination and lysis. Samples of red cells which had been treated with " blocking " Rhesus antibody (anti-D and anti-CD), of the cells of an infant suffering from erythroblastosis foetalis, and also of the cells of a case of atypical haemolytic anaemia (Boorman, Dodd, and Loutit, 1946) were included in the investigations and gave similar results.

Apparatus and Material

The cell used for the electrolysis consisted of a plastic (" perspex ") slide, in the centre of which was cut a circle of 12 mm. diameter and 4 mm. in depth. Into this were inserted two platinum wire electrodes, the ends of which were 5 mm. apart (Fig. 1). The wire was 0.02 S.W.G.

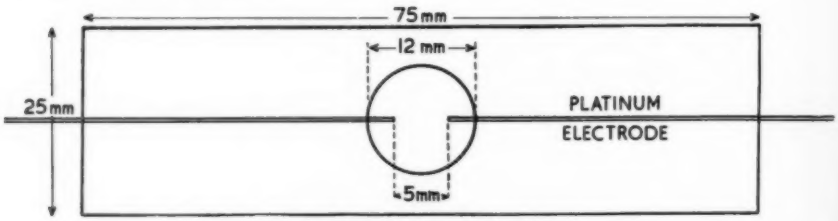

FIG. 1.—Line drawing of electric cell.

The cell was always completely filled with fluid when in use. A simple electrical circuit, incorporating a milliammeter and a variable resistance of 10,000 ohms in series with a 4½-volt high capacity dry battery (three Ever Ready dry cells high E.M.F.), was used.

Recently drawn red cells were used either from capillary blood suspended in saline or from Wintrobe oxalate vials.

Methods

Blood, 0.25 ml., was suspended in 5 ml. of isotonic saline and washed three times. In our experiments it was found that albumin inhibited the reaction.) After the third ashing sufficient saline was added to give a suspension of approximately 10,000 to 0,000 cells per c.mm. The suspension so obtained was examined qualitatively or uantitatively.

Qualitative Examination.—Part of the suspension so prepared was placed in the lectric cell and a current of 1 milliamp passed through it for three minutes. Following his a small amount of the suspension was removed by means of a Pasteur pipette and xamined microscopically on a slide for the presence or absence of agglutination. Under hese circumstances agglutination of normal red cells was readily seen.

Quantitative Examination.—Part of the suspension prepared as above was put into a ounting chamber and the number of cells per unit volume estimated. At the same time further sample of the suspension was put into the electric cell and a current of milliamp was passed through it for three minutes. After this time had expired the urrent was switched off and the number of cells remaining neither agglutinated nor sed were counted. The percentage of free cells could thus be estimated on a quantitative asis.

Results

Qualitative.—Twenty-five specimens of normal blood all underwent agglutination after three minutes.

Twenty experiments performed on normal red cells "blocked" *in vitro* all showed inhibition of agglutination and of lysis.

Twenty observations were made using the red cell of a newborn baby suffering from haemolytic disease of the newborn. On each occasion agglutination and haemolysis were inhibited.

Thirty experiments were made using the red cells of a case of mild atypical haemolytic anaemia. Again agglutination and haemolysis were inhibited.

Ten experiments were performed on sheep cells sensitized by rabbit anti-sheep haemolytic amboceptor. Agglutination and haemolysis were inhibited when compared with non-sensitized sheep cells.

Quantitative.—These results are presented in Table I.

TABLE I

THE QUANTITATIVE ESTIMATION OF THE AGGLUTINATION AND HAEMOLYSIS OF THE RED CELLS

Nature of Cells	Number of Experiments	Range of Free Cells (%)	Mean of Free Cells (%)	Standard Deviation	Value of t
Normal	25	30–53	45.0	6.6	—
Normal cells blocked *in vitro* ..	20	55–81	60.0	7.5	7.02
Erythroblastosis foetalis	20	55–83	63.7	8.4	8.5
Atypical haemolytic anaemia ..	20	55–78	63.2	5.8	9.3

On comparing the normal figures with each of the abnormal groups of results, a significant result for t was obtained. It will be seen from Table I that in 85

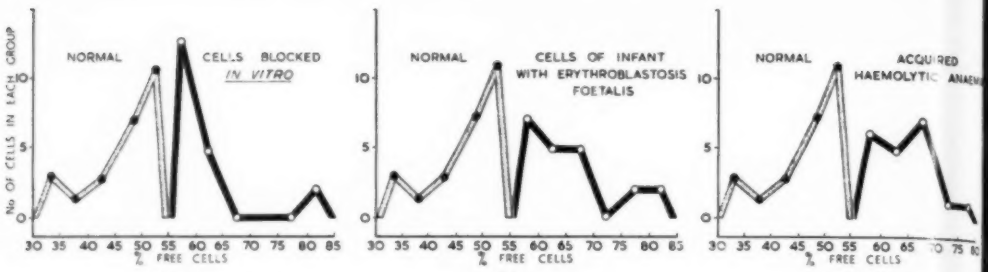

FIG. 2 FIG. 3 FIG. 4

Graphs to show the distribution of free cells arranged in groups of 5%.

paired estimations the percentage value of "free cells" was never more than 53. The percentage of free cells in the case of "blocked" cells was never less than 55. These results are shown graphically in Figs. 2, 3, and 4.

Discussion

Only two references in the literature have been found which deal with the effect of direct electric currents causing agglutination and lysis of red cells, and one of these, that of Petropavlovskiy (1935) is not available in this country. Its title "Electrolysis of Erythrocytes: Effect of Constant Current on Agglutination and Haemolysis of Horse's Erythrocytes" makes it clear that the author interested himself in this problem. The second (Mori, 1935) is written in Japanese, and there is a short author's summary in English, from which it is clear that he observed lysis of normal red cells in saline. Coombs and Race (1945) studied the electrophoretic mobility of red cells in a capillary tube through which a direct electric current was passing. They noted that the mobility of the cells was decreased if they had been previously treated with either "saline" or "albumin" Rhesus antibody. They made no report of agglutination or lysis of the cells. Mori suggested in his paper that the lysis resulted from the formation of alkali produced in the neighbourhood of the negative pole. In experiments performed on bacteria (Mudd, 1934; Abramson, Moyer, and Gorin, 1942) it was found that when organisms were suspended in an electrophoretic cell they migrated to one of the poles, but the addition of protein inhibited this movement. The fact that agglutination of red blood cells occurs in the presence of a direct electrical current is not easy to explain. It seems likely, however, that the coating of antibody known to be present on the outside of a sensitized red cell causes the inhibition of agglutination that we noticed in our experiments. It would appear that the method described is a delicate test for the presence of agents modifying the surface of red blood cells.

Summary

A method is described for the investigation of the effect of a direct electric current on red cells in saline.

Agglutination and lysis were observed. Partial inhibition of these phenomena occurred when sensitized red cells were used.

The fact that albumin inhibited the process suggested that protein on the outside of the erythrocytes caused some change in their response.

The method was found to give markedly abnormal findings in a case of atypical haemolytic anaemia that showed at times only a weakly positive Coombs reaction.

We would like to thank Professor R. J. V. Pulvertaft for the very helpful advice and criticism he has given us.

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THE EFFECT OF CITROVORUM FACTOR (FOLINIC ACID) ON MEGALOBLASTS *IN VITRO*

BY

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Sauberlich and Baumann (1948) found that an unidentified factor present in refined liver extract was necessary for the growth of the organism *Leuconostoc citrovorum* 8081. The addition of large amounts of folic acid to the culture medium was shown to produce slow growth in the absence of liver extract, suggesting some relationship between folic acid and the unidentified citrovorum factor (C.F.). Since then evidence has gradually accumulated that the citrovorum factor or factors are biologically more active derivatives of folic acid. The citrovorum factor also appears to be identical with folinic acid, a naturally occurring substance found by Bardos, Bond, Humphreys, and Shive (1949) to support growth of *Leuconostoc citrovorum* under the conditions described by Sauberlich and Baumann.

The citrovorum factor is about a million times more active for *Leuconostoc citrovorum* than is folic acid (Shive, Bardos, Bond, and Rogers, 1950) and is far more effective than folic acid in the competitive reversal of the toxicity of folic acid antagonists for *L. casei* (Bond, Bardos, Sibley, and Shive, 1949), rats (Nichol and Welch, 1950a), and mice (Broquist, Stokstad, and Jukes, 1950; Brockman, Roth, Broquist, Hultquist, Smith, Fahrenbach, Cosulich, Parker, Stokstad, and Jukes, 1950). In the treatment of the megaloblastic anaemia of monkeys produced by dietary deficiency C.F. has been shown to be active in much smaller doses than folic acid (May, Sundberg, and Schaar, 1950). The increased C.F. excretion in the urine of folic-acid-deficient rats and normal man after folic acid intake suggests an *in vivo* conversion of folic to folinic acid (Sauberlich, 1949). Liver slices will bring about a similar conversion *in vitro* (Nichol and Welch, 1950b).

In experiments on bone marrow cultures from cases of untreated pernicious anaemia folic acid has been found to act directly on megaloblasts producing ripening of these cells to normoblasts (Rusznyák, Löwinger, and Lajtha, 1948; Lajtha, 1950). The evidence that C.F. is a biologically more active form of folic acid suggested further *in vitro* studies to determine whether megaloblasts can utilize the citrovorum factor.

Methods

Suspensions of bone marrow cells were cultured in a medium of 90% serum and 10% Ringer solution, both cells and sera being obtained from patients with untreated

pernicious anaemia. To some cultures C.F. was added in a concentration of 0.1 or 0.01 μg. per ml. medium; to others, refined liver extract ("anahaemin") which had been assayed microbiologically for C.F. equivalent. Two samples of liver extract were used, one having a C.F. equivalent of 0.3 μg. per ml., the other 0.2 μg. per ml. Of the first extract 0.03 ml. was added to each ml. culture medium, i.e., the same volume as the "pernaemon forte" liver extract previously found to produce ripening of megaloblasts *in vitro* (Lajtha, 1950) giving a C.F. equivalent of 0.01 μg. per ml. medium. Of the second extract 0.03 ml. of a 1 in 100 dilution was added to each ml. medium, this concentration being chosen as being near to the pharmacological dose in untreated pernicious anaemia. This gave a C.F. equivalent of 0.07 μμg. per ml. medium

In two experiments the ripening effect of folic acid in a concentration of 0.1 μg. and 0.01 μg. per ml. medium respectively was compared with the effect of the C.F. at the same level. After 48 hours (in one experiment after 24 hours) films of the marrow cultures were stained and differential counts made on the nucleated red cells by each of us independently.

The results of the experiments are shown in Tables I and II.

TABLE I

THE EFFECT OF FOLINIC ACID ON MEGALOBLASTS *in vitro*

Experiment No.	Megaloblasts per 100 Nucleated Red Cells				
	Initial State	48-Hour Cultures in			
		P.A. Serum	P.A. Serum + 0.1 μg. C.F./ml.	P.A. Serum + 0.01 μg. C.F./ml.	P.A. Serum + 0.07 μμg. C.F./ml.
81a	32.0	35.0	14.0		38.0* 33.0*
81b	32.0	63.0	20.0		
82	36.0	31.5	16.5		
84a	42.0	29.5	14.0	15.0	
85	44.0	17.5	9.5	8.5*	
86	44.0	19.0			20.0

* C.F. equivalent in "anahaemin."

TABLE II

COMPARISON BETWEEN FOLIC ACID AND FOLINIC ACID ACTION *in vitro*

Experiment No.	Megaloblasts per 100 Nucleated Red Cells				
	Initial State	Cultures in			
		P.A. Serum	P.A. Serum + Folic Acid	P.A. Serum + C.F.	Remarks
84a	42.0		43.0	26.5	0.1 μg. medium. 24 hr. culture
85	44.0	17.5	19.5	8.5	0.01 μg. medium. 48 hr. culture

Discussion

It is clear that the C.F. has a similar ripening effect on megaloblasts in marrow culture to that found in previous experiments with folic acid. It also is able to overcome the action of the inhibitor present in serum from untreated pernicious

anaemia (Lajtha, 1950; Thompson, 1950). The effective concentration of folic acid used before was 2 μg. per ml. medium. In the present experiments 1/20 and 1/200 of this amount appeared to be ineffective. In contrast, similar concentrations, i.e., 0.1 and 0.01 μg. per ml. medium, of the C.F. showed a significant ripening effect. This provides further evidence that the C.F. is a biologically more active form of folic acid.

The previous finding that 0.03 ml. of a 1947 batch of refined liver extract ("pernaemon forte," Organon) ripened megaloblasts *in vitro* (Lajtha, 1950) now requires reconsideration. The effect could not be due to B_{12} since crystalline B_{12} was found to be inactive *in vitro*. The possibility that B_{12} in liver extract was modified in some way, giving a haemopoietic factor active *in vitro* similar to that found in normal serum, was suggested. There is good evidence for the existence of such a form of B_{12}, but the processes involved in the preparation of liver extracts would destroy its activity (Callender and Lajtha, to be published). Alternatively the ripening effect could have been due to the presence of folic acid or the C.F. None of the batch of "pernaemon forte" is available for assay for these substances. Another refined liver extract ("anahaemin") has, however, been tested. The folic acid content of the "anahaemin" was negligible, but the C.F. equivalent in 0.03 ml. of extract was 0.01 μg., which was sufficient in 1 ml. of medium to produce ripening of megaloblasts. At a lower concentration (C.F. equivalent 0.02 $\mu\mu$g. per ml. medium) closer to the estimated pharmacological dose of liver extract, no ripening occurred. As pure C.F. this level was also ineffective. This suggests that the effect previously noted with "pernaemon forte" was due to the C.F. content of the extract.

Summary

Pure citrovorum factor has a direct ripening effect on megaloblasts in bone marrow culture.

Weight for weight citrovorum factor appears to be more effective than folic acid in ripening megaloblasts *in vitro*.

We are indebted to Dr. T. H. Jukes, of the Lederle Research Laboratories, U.S.A., for supplies of the citrovorum factor and to Dr. G. Spray, Oxford, for the microbiological assays.

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THE RETICULOCYTE CRISIS AFTER THE EFFECTIVE SPECIFIC TREATMENT OF PERNICIOUS ANAEMIA

BY

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Since liver extracts began to be used in the treatment of pernicious anaemia nimated discussion has been carried on with regard to the cause of the reticulocyte isis that then occurs. The phenomenon is by no means easy to explain, and at rst sight it would seem absurd that following effective treatment of pernicious naemia only a sudden, transient increase in the reticulocytes can be obtained, espite the fact that the red blood cells subsequently continue to rise to normal alues.

The simplest way of getting over the difficulty is to assume, as many investiators have already done, that the remission in pernicious anaemia takes place ithout the newly formed red blood cells passing through the normal reticulocyte age. No really conclusive proof of the correctness of this assumption has as yet en advanced.

After Singer, King, and Robin (1948) had reported their discovery that blood rpuscles transfused from untreated cases of pernicious anaemia to normal hildren had a much shorter survival time (28–75 days), but after treating the patients with a liver extract their newly formed red blood cells had a normal life cycle, the discussion was transferred to some extent to other aspects of the problem. It was then thought that the initial increase in the reticulocytes was due to enormous hyperactivity of the bone marrow following the treatment, and that a number of more or less defective blood cells were produced before and during the early remission and then rapidly eliminated in the peripheral circulation. After the production of red cells has begun to function effectively, however, more and more normal erythrocytes with a normal life cycle were formed and consequently there was no longer any need for such an intensive delivery of newly formed blood cells per time unit. This was the explanation advanced to account for the decrease in the reticulocytes. It is obvious, however, that this explanation is not satisfactory when it is subjected to a critical analysis. In pernicious anaemia it is the rule that no pronounced increase in the number of red blood corpuscles is observed in the reticulocyte crisis. It is only after some time has passed that the blood values begin to show a more or less steady tendency to rise, and the need of full activity in the blood-cell factories therefore seems undeniable. The more stable structure of the newly formed blood corpuscles, which would cause among other things a normal life span *in vivo*, could hardly be expected to make itself felt before—at the very

earliest—the time when the previously formed, defective blood corpuscles had completed their life cycle; in other words, according to the investigations of Singer *et al.*, until after 28 days. I have repeated Singer's experiment and was able to confirm his statements to the full; in one case, however, the life span of the megalocytes showed the relatively low figure of 18 days. Consequently, the change in the length of the life cycle definitely cannot explain the abrupt drop in the reticulocyte count. If blood from a pernicious anaemia patient during the upward swing of the reticulocyte crisis is transfused to a normal person (Berlin, 1950), the maturation curve for the reticulocytes *in vivo*, which is obtained by determining the reticulocyte value in the recipient's blood immediately after the transfusion and then at frequent intervals during the subsequent 24 hours, will be as shown (Fig. 1*a*). If the blood is transfused just at the time when the reticulocyte peak is reached another type of curve is obtained (Fig. 1*b*), and if the blood is taken after the crisis is over and the reticulocytes are on the decrease again the maturation curve will have still another appearance (Fig. 1*c*). It will be seen that the total maturation time, roughly 17–18 hours, is surprisingly similar in all three instances. The course of the curve in Fig. 1*a* may be explained by the fact that the distribution of cells of different ages among the transfused reticulocyte population was uneven, and that older, more mature cell-types were in the majority. After a short time the older forms developed rapidly after entering a normal environment, and the final, more horizontal part of the curve in all probability shows the maturation of the youngest, newly liberated reticulocyte forms which later on developed in the recipient's circulation within the normal time, i.e., 18 hours. Fig. 1*b*, on

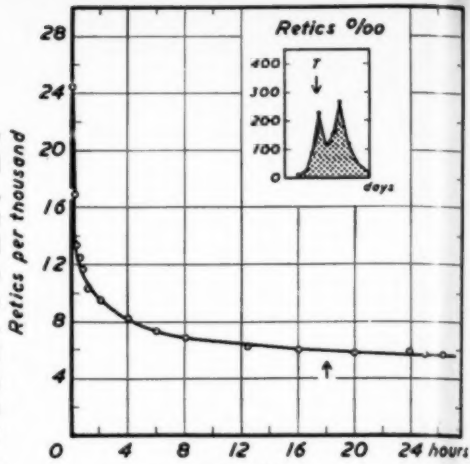

FIG. 1 (*a*)

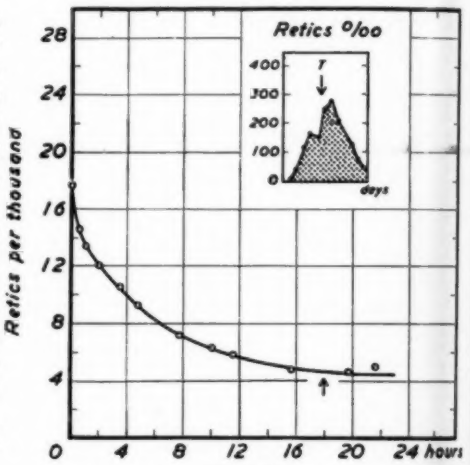

FIG. 1 (*b*)

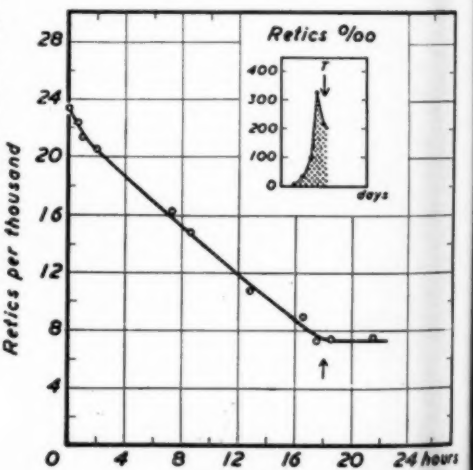

FIG. 1 (*c*)

e other hand, with a more even slope probably indicates that the different ages f the reticulocytes were more evenly distributed. The third type of curve ig. 1*c*) would seem to indicate that there was a preponderance of young, newly oduced forms among the transfused reticulocytes. To obtain accurate informa- n 10,000 cells have been counted on every slide. The standard deviation in these lculations has not exceeded 8% of the mean. On the basis of these experimental servations the supposition might be advanced that the maturation time of ticulocytes at the start of the reticulocyte peak is considerably prolonged ; at that age relatively old, more highly developed reticulocytes are over-represented—in tients with pernicious anaemia—but the maturation process rapidly begins function in the recipient's normal environment. After the reticulocyte peak e conditions would be the reverse, the maturation of the reticulocytes now pro- eds more rapidly, and consequently a sudden drop in the number of reticulo- tes is found, and the cells consist, for the most part, of newly formed immature rms.

It is surprising how little attention has been paid to the investigations Jörgensen and Plum (1943) and Ruth Plum (1947) into the maturation of iculocytes. These authors have demonstrated that there is a special reticulocyte pening factor in the blood, and they have made a study of this factor under normal d pathological conditions. They point out that in untreated pernicious anaemia tients there is a considerably lowered amount of the active ripening factor in sma, but during effective treatment this increases not only to normal values but en to values exceeding the normal figures. They have also shown that consider- ble amounts of the ripening factor can be found in pernicious anaemia patients t that it occurs in an inactive form as long as the disease remains untreated. ese observations throw further light on the interpretation of the *in vivo* reticulo- yte-maturation curves and strengthen the assumption that at the beginning of the reticulocyte crisis there is increased erythropoiesis, set in motion by the liver therapy, but that the increase, or activation, of the reticulocyte-ripening factor, which should follow the treatment, has not yet come fully into action. The result will then be that the newly formed blood corpuscles remain at the reticulocyte stage for a longer time than normally, perhaps for several days. Consequently, there must be a considerable increase in the number of reticulocytes in the peri- pheral blood, and the majority will be relatively old cells. When the curve has reached its peak the amount of substance able to transform reticulocytes into mature red cells has, however, increased in the blood, the previously older cells develop rapidly into non-reticulated cells, and the newly formed reticulocytes also quickly develop. There is then a steep drop in the curve, which corresponds to a decrease in the reticulocytes. During the entire remission the maturation factor is present in normal or more than normal amounts, and, despite the fact that erythropoiesis is still very lively, the reticulocytes do not increase very much above the normal figure. It is thus unnecessary to have recourse to the unverified statement that the cells produced as the remission proceeds are liberated from the bone marrow without passing through the reticulocyte stage. This reasoning also receives support from the well-known observation, that, if a pernicious anaemia patient is given a suboptimal dose of liver extract so that a pronounced reticulocyte response is obtained but no real remission starts,

the blood values remain constant, the serum iron level tends to rise, and the sternal marrow begins, in some cases, to display megaloblast proliferation: when effective treatment is instituted later the reticulocyte response is extremely slight. In accordance with this line of reasoning, there would then still be enough maturation factor left, and consequently the reticulocyte crisis could not develop. These results would explain the findings of Davidson, Murphy, Watson, and Castle (1946), who found that a patient with pernicious anaemia, transfused to bring the blood up to normal levels, failed to produce a reticulocyte crisis when given liver extract. This has previously been interpreted as being due to the fact that, by bringing the patient's blood up to nearly normal values, the new formation of red blood cells was hindered. It cannot be denied, however, that this result might just as well be a consequence of the fact that with the transfused blood such large amounts of reticulocyte-ripening factor had been supplied that it had sufficed to prevent the occurrence of a reticulocyte crisis in response to the subsequent liver therapy.

Summary

The current views regarding the mode of origin of the reticulocyte crisis following liver treatment in pernicious anaemia is analysed. Experiments are described which show that, if blood from pernicious anaemia patients is transfused during the reticulocyte crisis to normal persons under different conditions, the curves of the reticulocyte development that are obtained by determining the number of reticulocytes in the recipient's blood at frequent intervals after the transfusion will also be quite different. It is considered probable that when blood cells are transfused at the beginning of the reticulocyte crisis there will be a rapid initial development of a high percentage of older reticulocytes. If, on the other hand, the blood is transfused when the reticulocyte peak is on the wane, the drop noted in the maturation curve suggests that in this instance the population is chiefly of young, immature cells.

These observations are compared with the investigations into the reticulocyte-ripening factor occurring in pernicious anaemia reported by Jörgensen and Plum and by Ruth Plum; these authors found a reduced amount of active factor in untreated cases of pernicious anaemia. The amount of this substance in plasma rises to values almost higher than normal during the reticulocyte crisis.

It is thought that the change in the development rate of the reticulocytes at the beginning and at the end of the reticulocyte crisis in all probability is the explanation of this otherwise incomprehensible phenomenon.

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THE SAMPLING ERROR IN HAEMOGLOBIN DETERMINATION

BY

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Of the numerous methods for the estimation of haemoglobin the one which gives the most generally reliable results is that using oxyhaemoglobin in the Medical Research Council neutral grey wedge photometer (Macfarlane, King, Wootton, and Gilchrist, 1948; King, Wootton, Donaldson, Sisson, and Macfarlane, 1948). An experiment using this method at various levels of haemoglobin showed that in a routine laboratory it gave results on single samples of blood with an average standard deviation of 1.6% of haemoglobin (see experiment I). This remarkably small technical error suggests that a difference of 5% between two readings on one patient would usually indicate that a real change in haemoglobin level had occurred. In other words, if two readings on one patient were 100 and 95% on 19 out of 20 occasions, these readings would indicate a real change in haemoglobin level.

In the routine use of the M.R.C. photometer it is soon apparent that this precision is not reached. Much larger differences in haemoglobin than 5% may result from random variation. This increased variability is not due to technical inaccuracy but to the differences between two samples of blood. The haemoglobin level of the blood in one normal person is not a static and unchanging figure. The distribution of cells and plasma in different 0.02-ml. samples taken from the same person at the same time is not constant. The haemoglobin level changes during the course of the day and from one day to another. This inherent variability in the haemoglobin readings of normal people cannot be distinguished by the clinician from purely technical errors. The clinician wishes to know whether a difference between two readings on a single patient may be attributed with reasonable certainty to the progress of disease or the effects of treatment. The fact that the haemoglobin level of normal people is not constant must be taken into account in assessing the results of the test.

The variability of haemoglobin readings in normal people is well recognized. In 1941 Sörensen showed that samples of capillary blood taken at the same time had a different haemoglobin content and that the variation was greater with blood from the ear than that from the finger. McCarthy and Van Slyke (1939), Mole (1945), Renbourn (1947), and others all showed that the haemoglobin level in normal people varies through the day. In most people there is a progressive fall in haemoglobin between 6 a.m. and 10 p.m. and a rise during sleep. Between 9 a.m. and 5 p.m. when routine blood samples will usually be taken, the change is not large, amounting to a change of 2 to 3% of haemoglobin. Renbourn (1947) showed that

the haemoglobin level of normal people varies from one day to another independently of any diurnal variation and that this daily variation is larger than the diurnal changes.

With the introduction of a reliable method for haemoglobin determination into routine use it is important to recognize the extent of the normal fluctuation in readings to be expected, and to determine whether or not this source of variation can be reduced, for example, by the use of venous blood samples for haemoglobin estimations. Experiments were undertaken to determine the probable extent of natural variation in haemoglobin.

Methods

Collection of Blood.—Heparinized venous blood samples and capillary blood samples from the finger were used.

Haemoglobin Determination.—Whole blood, 0.02 ml., is pipetted into 4 ml. of ammoniated distilled water and the level of haemoglobin is read with the M.R.C. neutral grey photometer. This instrument is graduated in percentage of haemoglobin taking 100% to represent 14.8 g. in 100 ml. of blood.

Haematocrit.—The microtechnique for the determination of packed cell volume was worked out in collaboration with Dr. Marmion.

Anticoagulant.—About 0.05 g. of dried heparin is ground in a mortar with 5 ml. of refined liquid paraffin. The suspension is thoroughly shaken before use because the heparin sediments to the bottom.

Haematocrit Tubes.—Uniform bore, thick-walled barometer tubing is cut into sections about 4 in. in length. These are ground to a tapered point at one end. The internal diameter of the tubing is about 0.8 mm.

Technique.—Immediately before use the anticoagulant is drawn into the tube and blown out to leave a coating on the internal surface. Blood is drawn up to an arbitrary level and below this a short column of mercury is drawn up to seal the lower end. The tube is encircled with a stout rubber band and enclosed in a test-tube with a rubber buffer in the bottom, and is centrifuged at 3,000 r.p.m. for 30 minutes. The paraffin separates as a layer on top, and the length of the column of packed cells and plasma can be measured.

With one sample of venous blood this method gives a coefficient of variation of about 1.5%.

Experimental Results

Experiment I: The Technical Error of the M.R.C. Photometer.—Four separate experiments were made using a range of different haemoglobin pipettes and employing five observers to make the readings. In each experiment 50 subsamples of 0.02 ml. of blood were taken from one venous blood sample and the level of haemoglobin was measured. The average haemoglobin levels of these samples were 98.1%, 69.8%, 32.0%, and 17.2%. In these experiments the total variability of the readings was represented by standard deviations of 1.66, 1.37, 2.04, and 1.28% of haemoglobin.

The extent of technical variability, including differences between observers, was remarkably small, having an average value of 1.59% of haemoglobin. An interesting feature of this technical error was that it appeared to be independent of the level of haemoglobin. At low levels of haemoglobin therefore the technical error is relatively

more important; at 17% of haemoglobin the technical error gave a proportional error (coefficient of variation) of 7.44%.

Experiment II: The Variability of Haemoglobin Readings on Capillary Blood Samples Taken at One Time—Since the technical error of the method does not account for the variability of haemoglobin readings in practice, an attempt was made to see whether or not the variability could be attributed to differences between capillary blood samples. Four experiments were carried out. In each of these 20 samples of capillary blood were taken in rapid succession from one normal individual using 20 different pipettes. The haemoglobin levels in the diluted samples were read by two observers. The mean haemoglobin levels and the total standard deviations for each experiment are shown in Table I. It is obvious that an important

TABLE I

MEAN HAEMOGLOBIN LEVEL AND TOTAL STANDARD DEVIATION AND COEFFICIENT OF VARIATION IN EXPERIMENT II

Experiment No.	Mean Haemoglobin (%)	Standard Deviation (%) of Haemoglobin	Coefficient of Variation
IIa	96.4	4.09	4.24
IIb	105.3	2.60	2.47
IIc	100.5	5.35	5.32
IId	88.4	5.98	6.76

increase in variability is introduced by the use of capillary samples. Whereas the mean technical error was 1.6% of haemoglobin, that for the experiments using capillary blood was 4.5%.

Experiment III: The Variability of Haemoglobin Readings on Capillary Blood Samples Taken on Different Days.—To extend the results of experiment II haemoglobin readings were made on 20 normal subjects and 20 patients on six or seven successive days. In each normal individual or patient tested five capillary samples were taken at the same time on each day. In this way a total of 30 or 35 estimations were made for each subject. The levels of haemoglobin in the patients covered a wide range from 23 to 100%. In the normal people the range was from 83 to 120%. In this way it was hoped that it might be possible to make an approximate estimate of the extent of random variability to be expected at different haemoglobin levels. In the normal people there was no consistent trend of haemoglobin change from one day to another, but in many of the patients an increase or decrease in haemoglobin occurred over the test period. In the anaemic patients an improvement followed appropriate treatment. In seven patients in the puerperium there was a rise in haemoglobin presumably due to readjustment of the blood volume following delivery. In a patient with aplastic anaemia there was a fall in haemoglobin associated with the failure of normal erythropoiesis. Since the experiment was designed to assess the random variation to be expected from capillary haemoglobin samples the influence of these consistent trends in haemoglobin levels had to be eliminated from the results.

The results were calculated for each individual by analysis of variance. In this way the error due to variations in haemoglobin from day to day could be separated

from random variability. The variation from one day to another was then separated into two components, one that could be attributed to linear regression, i.e., the consistent change in haemoglobin level, and one due to deviations from the regression or random variations from one day to another. The components of random variation were combined to give the total error. An example of the calculations involved is given in Table II.

In nine subjects five microhaematocrit readings were made on each day of the experiment. The haematocrit method has a small technical error, and the results

TABLE II

THE HAEMOGLOBIN LEVELS EXPRESSED AS PERCENTAGES OBSERVED IN ONE PATIENT IN EXPERIMENT III AND THE CALCULATIONS TO DETERMINE THE TOTAL OF RANDOM ERROR

Sample	Day of Experiment 1	2	3	4	5	6	7
1	38	48	49	51	47	48	60
2	40	45	47	52	50	51	60
3	42	47	50	54	51	51	61
4	45	43	52	48	51	52	60
5	46	44	49	52	50	53	58
Total	211	227	247	257	249	255	299

Sum = 1745 mean = 49.86
n= 35

Total sum of squares

$$= (38^2 + 40^2 - 60^2 + 58^2) - \frac{(1745)^2}{35}$$

$$= 1030.29$$

Sum of squares referable to differences between days

$$= 1/5(211^2 + 227^2 - 299^2) - \frac{(1745)^2}{35}$$

$$= 906.29$$

Sum of squares due to linear regression calculated from the formula

$$\frac{(Sxy - \frac{SxSy}{n})^2}{Sx^2 - \frac{(Sx)^2}{n}}$$

$$= 740.60$$

Deviation from regression = 906.29 − 740.60
= 165.69

ANALYSIS OF VARIANCE

Source of Error	D.F.	Sum of Squares	Mean Square	Standard Deviation
Total	34	1030.29		3.30
Regression	1	740.60		
Deviations from regression	5	165.69	33.14	2.40
Random error	28	124.00	4.43	2.10

of the test should give readings which vary in the same way as the haemoglobin readings. These additional tests were carried out to ensure that the variation in haemoglobin readings could not be attributed to some unidentified technical error in the haemoglobin method. The haematocrit values showed day-to-day variations parallel to the haemoglobin readings (Fig. 1), and the random variability, to be attributed to sampling errors, was similar to that of the haemoglobin estimations. The observed variations in haemoglobin percentage must therefore be attributed to differences between capillary samples and random changes in haemoglobin from one day to another.

The extent of the observed variation was wide, ranging from a standard deviation of 1.65 to 6.96% of haemoglobin. Expressed as a percentage of the mean haemoglobin readings (coefficient of variation) the error tended to increase with lower levels of haemoglobin (Fig. 2). Fig. 2 shows that there is no close correlation between the extent of the

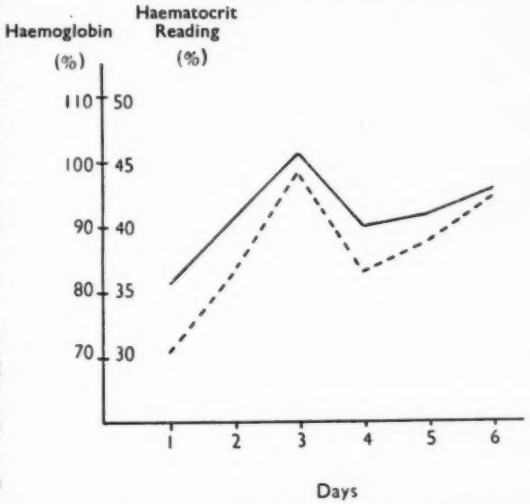

FIG. 1.—The haemoglobin readings of a patient in the puerperium as a mean value for five daily estimations made on six consecutive days (solid line). The haematocrit values as a mean of five estimations made on the same six days and at the same time as the haemoglobin readings (broken line).

TABLE III

ERROR IN ESTIMATING PERCENTAGE OF HAEMOGLOBIN USING THE M.R.C. NEUTRAL GREY WEDGE PHOTOMETER

Haemoglobin Level (%)	Calculated Range of 19/20 Observations on One Individual	Significant Difference (% Hb)
120	113–127	10
110	102–118	11
100	91–109	12
90	81–99	13
80	71–89	13
70	61–79	13
60	51–69	12
50	42–58	11
40	33–47	10
30	24–36	8
20	16–24	6

In the second column is indicated the range within which separate observations on one individual should fall. In the third column is shown the difference between two isolated haemoglobin readings on one person which would suggest that a real change had occurred (calculated from the formula $2\sqrt{2S^2}$)

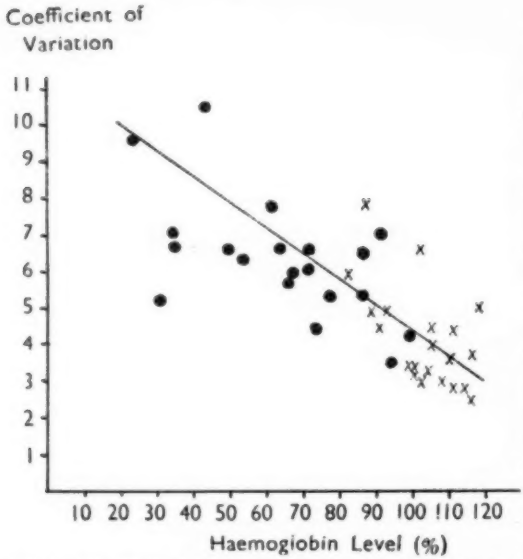

FIG. 2.—The coefficients of variation for the haemoglobin readings of the patients and normal people tested in experiment III plotted against the level of haemoglobin. (Experiments with normal individuals, X. Experiments with patients, ●).

error and the haemoglobin level but that the error does tend to increase with the lower values. This increase in error is due in part to the technical error which appears to be constant at different haemoglobin levels and, though small, will become proportionately large at low values of haemoglobin (see experiment I).

It is clear from Fig. 2 that no exact limits of variability to be expected in routine haemoglobin readings can be laid down, but as a rough guide to the general order of these changes an approximate mean value for the coefficient of variation at different haemoglobin readings was obtained from Fig. 2 and from these the figures in Table III were obtained. The limits of variability given in Table III correspond reasonably well with the random fluctuations in haemoglobin encountered in the routine laboratory.

Experiment IV: The Variability of Venous and Capillary Blood Samples on Different Days.—It seemed possible that some variability might be a feature of capillary blood and that venous blood might be more consistent in its constituents. An experiment was designed to compare the variability of venous and capillary samples on different days.

In this experiment two 0.02-ml. samples of capillary blood and one venous sample were taken from five normal subjects on ten consecutive days. The errors

TABLE IV

VARIABILITY OF HAEMOGLOBIN READINGS OF VENOUS AND CAPILLARY BLOOD

	Day-to-day Variation (%)	Random Variation (%)	Total Variation (%)
Error determined from capillary blood samples ..	4.40	4.05	6.01
Error determined from venous blood samples ..	4.62	3.65	5.83
Error of microhaematocrit	4.30	4.50	6.29
Error of M.C.H.C.	1.91	3.85	4.30

expressed as coefficients of variation calculated by analysis of variance are given in Table IV. The variability of the capillary and venous samples was similar, and each showed a significant difference from day to day.

In addition the packed cell volume was measured by the micro-method on both venous and capillary blood for six days of this experiment. The packed cell volume showed a variability similar to that of the haemoglobin. As would be expected the mean corpuscular haemoglobin concentration showed a random variation similar to that of haematocrit and haemoglobin estimations, but there was little variation from one day to another. This experiment shows that the differences between venous samples are as great as those between samples derived from capillary blood. There can be no advantage in using venous blood samples for haemoglobin determination. For the estimation of the mean corpuscular haemoglobin concentration it is obviously advisable to use one sample of venous blood. In this way the random variability of different samples will be avoided.

The Diurnal Variation in Haemoglobin.—The extent of the diurnal variation in haemoglobin for the hours during which samples of blood are taken for examination in the routine laboratory is not large (Renbourn, 1947). The labour involved in taking serial blood samples at the same time of day would be great and would not increase the precision of the method noticeably.

Summary

These experiments have shown that the variation in haemoglobin readings to be expected in the routine use of the M.R.C. neutral grey photometer is much greater than that which can be attributed to the technical error of the method. This increased variability is due to differences between one capillary blood sample and another and to changes in the level of haemoglobin from one day to another. No reduction in error can follow the use of venous blood samples because venous samples show differences as great as those between capillary samples. The increased precision which might follow the collection of serial samples at one time of day is small in relation to the other unavoidable errors. If it is desired to determine the mean corpuscular haemoglobin concentration the haemoglobin and haematocrit readings should be made on the same sample of venous blood, when sampling errors will be avoided. In the routine use of the M.R.C. photometer the errors to be expected at different haemoglobin levels are shown in Table III. These figures are compiled from the results of experiment II and give an approximate guide to the general variability of the method.

We should like to thank all the volunteers who took part in the experiment. We should also like to thank Dr. R. G. Macfarlane for his advice and interest in the problem, and Mr. Finney, who kindly read the manuscript.

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THE SURVIVAL OF TRANSFUSED ERYTHROCYTES FROM PATIENTS WITH POLYCYTHAEMIA VERA

BY

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The following observations are concerned with the survival of erythrocytes from three polycythaemic patients transfused into normal recipients and estimated by the method of differential agglutination.

The donors were (1) a man aged 60, known to be suffering from polycythaemia vera for seven years. A typical blood count at one time in July, 1949, gave R.B.C. 11.6 million, W.B.C. 19,000, Hb 112% (Haldane 100% = 14.8 g.%), and a haematocrit of 76 ; (2) a man aged 34, first seen in January, 1949, with the follow-

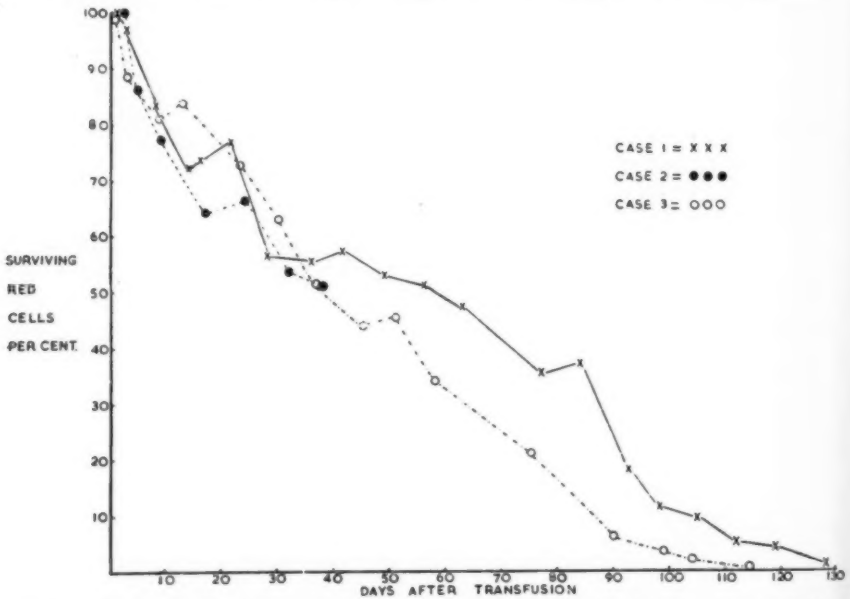

FIG. 1.—Diagram showing survival of cells from cases of primary polycythaemia after transfusion, into normal recipients.

ing blood count: R.B.C. 11 million, W.B.C. 17,000, Hb 156%, and a haematocrit of 84; (3) a man aged 61, first seen in March, 1950, when he had a blood count of R.B.C. 8.7 million, W.B.C. 81,000, Hb 126%, haematocrit 58, and was found to have a myelosclerotic bone marrow. This latter association in certain cases of polycythaemia vera has recently been stressed by Dameshek (1950).

The recipients were (1) a normal man, aged 28; (2) a man aged 42, suffering from carcinoma of the bronchus, whose general condition was initially satisfactory, except for a slowly resolving paraplegia due to spinal secondaries; (3) a woman aged 62, recovering from a cerebral thrombosis and with mild iron deficiency anaemia. Unfortunately, six weeks after transfusion, the second recipient died from cerebral secondaries, which became manifest four weeks after transfusion.

Technique

Approximately 1 pint of citrated blood from a polycythaemic donor was transfused after storing for 24 hours into the recipient. The day after transfusion and at intervals thereafter the surviving cells were estimated by differential agglutination, using the ABO system. This was carried out by Dacie's method (1950).

For the purpose of constructing a graph the basal inagglutinable count was subtracted from the inagglutinable count found on any particular day after transfusion. That count on the day after transfusion is called 100% survival, and the number of surviving cells on subsequent days is expressed as a percentage of this figure.

TABLE I

SURVIVAL OF CELLS FROM CASES OF PRIMARY POLYCYTHAEMIA AFTER TRANSFUSION INTO NORMAL RECIPIENTS

Transfusion from Case 1 to Recipient 1*			Transfusion from Case 2 to Recipient 2†			Transfusion from Case 3 to Recipient 3‡		
Days after Transfusion	Observed Count (c.mm.)	Percentage Cells Surviving	Days after Transfusion	Observed Count (c.mm.)	Percentage Cells Surviving	Days after Transfusion	Observed Count (c.mm.)	Percentage Cells Surviving
1	570,000	100.0	2	480,000	100.0	1	679,000	100.0
3	555,000	97.3	5	422,000	86.7	3	607,600	89.3
8	480,000	84.0	9	382,000	77.6	9	558,800	81.9
14	417,000	72.7	17	325,000	64.6	13	566,400	83.1
16	423,000	73.8	24	335,000	66.9	23	504,200	73.9
22	442,000	77.1	32	278,000	53.9	30	447,000	63.6
28	328,000	56.9	38	267,000	51.2	37	359,000	51.9
36	322,000	55.8				45	319,000	44.4
42	330,000	57.4				51	326,000	45.4
49	306,000	53.0				58	245,400	34.7
56	299,000	51.7				75	139,700	21.0
63	275,000	47.4				90	59,200	6.8
77	210,000	35.9				99	39,200	3.8
84	216,000	37.0				104	32,800	2.8
93	112,000	18.4				114	19,200	0.8
98	75,000	11.8				119	13,400	0
105	61,800	9.5						
112	39,400	5.5						
119	36,000	4.9						
128	19,400	1.9						
140	7,600	0						

* Basal inagglutinable count 8,500/c mm. † Basal inagglutinable count 41,500/c mm.
‡ Basal inagglutinable count 13,700/c mm.

Results

The results are shown in Fig. 1 and Table I. The survival of cells from polycythaemic patients was found to be normal in the experiments which were completed. The rate of destruction in the patient who died six weeks after transfusion was also found to be normal.

Summary

Our observations show that the life span of transfused red cells from cases of primary polycythaemia is normal. These results confirm the findings of Merskey (1949), who used a similar differential agglutination technique. There is further confirmation that the red cell life in primary polycythaemia is normal. Using another technique (with isotopic nitrogen), London, Shemin, West, and Rittenberg (1949) have shown in one case that the otherwise undisturbed red cell in a polycythaemic patient has a normal life span.

We wish to thank Dr. A. E. Mourant for providing us with high titre agglutinating sera.

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COMBINED FRAGILITY TEST FOR CONGENITAL HAEMOLYTIC JAUNDICE

BY

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In congenital haemolytic jaundice or familial acholuric jaundice increased osmotic fragility is by no means always present. Many cases, otherwise typical, have been shown to have normal fragility (Lommel, 1913 ; Dawson, 1931 ; Hansen and Klein, 1934 ; and others), and Gänsslen (1937) estimates that these may constitute 10% of the total. In some cases where a normal fragility is found at first a slight increase of fragility may sometimes be encountered if the test is repeated on several occasions. On the other hand, patients who have previously shown grossly increased fragility may show normal or nearly normal values during haemolytic crises. Even when demonstrated, however, increased fragility is not diagnostic of congenital haemolytic jaundice, for many acquired haemolytic (haemolysinic) anaemias also show it.

It was thought that a study of the fragility distribution by the latest photo-electric methods might reveal abnormalities which would be characteristic for congenital haemolytic jaundice even when the "minimum osmotic resistance" was normal or nearly normal. An opportunity to test this possibility came when a family of 12 members, five of whom had reticulocyte counts greater than 5% in the absence of anaemia, was encountered. As controls, four normal subjects, three with simple iron deficiency anaemia, two with iron deficiency anaemia of pregnancy, and one with spherocytic anaemia occurring during pregnancy, were studied. Of the 12 members of the family, only five (four affected and one normal) could attend the laboratory: the others were visited at their homes, up to 170 miles away, and the samples brought by public transport to the laboratory. Thus a delay of up to 24 hours occurred before they were tested. Control samples were treated in the same way, and it was found that blood from individuals with congenital haemolytic jaundice showed a marked increase in fragility after standing for 24 hours, while that of normal and other anaemic subjects showed a much smaller increase, about 1/5 to 1/10 of that shown by the blood from congenital haemolytic jaundice.

Methods and Results

The haemoglobin content was determined by King's method standardized by haemin. Red cell counts were made in a dilution of 1/200, and 2×80 small squares were counted in a Bürker's chamber. Reticulocytes were determined by a wet method, and Price-Jones curves by projection (Humble and Belyavin, 1948) ; 200 cells were counted. Fragility

was tested by Discombe's (1948) modification of Hunter's method. All blood was heparinized.

The family tree is shown in Fig. 1, and haematological data in Tables I and II. A few fragility curves are given in Figs. 2–8. Control experiments showed that mechanical damage during transport was negligible.

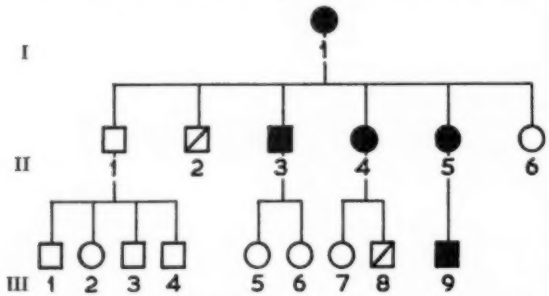

FIG. 1.—Individuals II 2 and III 8 dead, III 1 not examined. Circles = females, squares = males. Dark figures = affected individuals.

The three cases of congenital haemolytic jaundice examined immediately after venepuncture all showed normal values of mean cell fragility (M.C.F.), and, though the standard deviation was considerably increased, the minimum osmotic resistance was within the limits of normal. After 24 hours the M.C.F. had risen by about 0.2% NaCl, and the standard deviation had doubled, so that the osmotic resistance reached the grossly abnormal values expected in congenital haemolytic jaundice. None of the control cases showed such an increase, the increase in M.C.F. being 0.21% NaCl and the standard deviation showing only a slight change. It was interesting to find that the spherocytic anaemia of pregnancy differed so completely from that of congenital haemolytic jaundice.

Discussion

Many workers have observed that the blood of patients with congenital haemolytic jaundice undergoes an abnormally quick spontaneous haemolysis on standing (Lüdke, 1918 ; Meulengracht, 1922 ; Gripwall, 1938 ; Ham and Castle, 1940a ; Dacie, 1941 ; Lloyd, 1941 ; Caroli, Étévé, Paraf, and Robineau, 1949). Dacie noted that the speed of spontaneous haemolysis in congenital haemolytic jaundice only was associated with increased fragility, but that this was not true in acquired haemolytic anaemia. Three hypotheses have been suggested to account for this fact: first, the action of lysolecithin (Bergenhem and Fåhraeus, 1936 ; Gripwall, 1938) ; second, the abnormal shape of the cells (Ham and Castle, 1940a) ; and third, a structural defect in the cell (Lloyd, 1941).

Singer (1940) showed that the cells in congenital haemolytic jaundice were more susceptible to lysis by "lysolecithin" than were other cells, and claimed thus to differentiate the haemolytic anaemias.

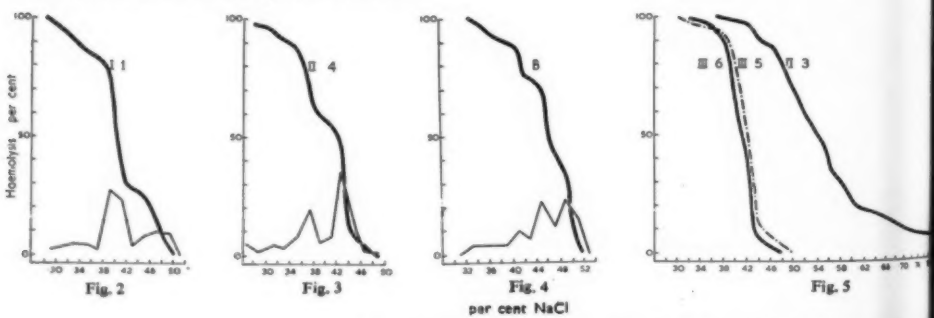

Fig. 2 Fig. 3 Fig. 4 Fig. 5

FIGS. 2–8.—The lower curves represent the fragility distribution, co

Caroli *et al.* (1949) showed that the normally spherocytic and osmotically fragile erythrocytes of the goat underwent spontaneous haemolysis no more rapidly than did normal human cells, thus showing that spherocytosis alone had nothing to do with spontaneous haemolysis, this observation being confirmed for human subjects with acquired spherocytosis by Dacie (1941).

Ham and Castle (1940a and b), Emerson, Shen, and Castle (1946), Emerson, Shen, Ham, and Castle (1947), and Young (1947) observed that red cells incubated at 37° C. had an increased osmotic fragility especially marked in congenital haemolytic jaundice. This was also the case where fresh cells did not exhibit a significant increase of fragility (Young, 1947). This modified fragility test appeared to be specific for congenital haemolytic jaundice in Young's series.

All these observations can be reconciled on the hypothesis that the red cell of congenital haemolytic jaundice is structurally abnormal, and that on standing it deteriorates more rapidly than normal cells. This abnormality can be demonstrated by the "lysolecithin" test, by determination of fragility after standing 24 hours, or by a study of the rate of spontaneous haemolysis. Increased osmotic fragility itself is a function of the degree of spherocytosis (Haden, 1934), and when present in fresh cells has nothing to do with any other structural abnormality; but when blood is allowed to stand, the fundamental structural abnormality of the red cells in congenital haemolytic jaundice causes a slow decrease in cell integrity, which can be detected after a few hours by the change in osmotic fragility and spontaneous haemolysis. This deterioration affects the whole red cell population, and can be detected earlier and with greater certainty by fragility measurements than by the observation of spontaneous haemolysis. Fragility measurements at 0 and 24 hours after taking blood are much simpler than the "lysolecithin" test, and might be referred to as a "combined fragility test."

Summary

Congenital haemolytic jaundice can occur without clinical manifestations, whereas changes in fragility are inconstant and often indistinguishable from those occurring in other forms of anaemia.

When blood from a patient with congenital haemolytic jaundice is stored for 24 hours at temperatures between 20 and 37° C. the fragility increases greatly, the mean cell fragility increasing by 0.15–0.2% NaCl and the standard deviation being doubled.

The increase in the mean cell fragility of blood from normal subjects and subjects with other anaemias is only about one-tenth as large.

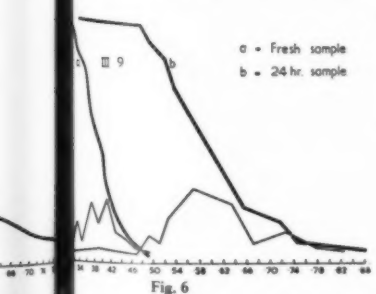

Fig. 6

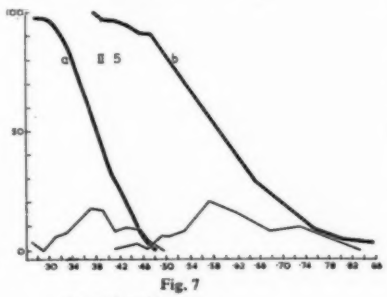

Fig. 7

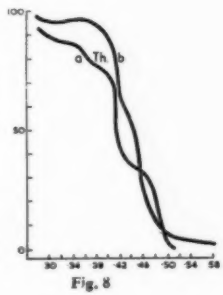

Fig. 8

per cent NaCl

ion, con e corresponding curves using the method of haemolytic increments.

Singer's "lysolecithin" test and the combined fragility test appear to be diagnostic of congenital haemolytic jaundice. The latter is more convenient.

The combined fragility test is likely to be of value in studying "healthy carriers" and in cases where the clinical manifestations are slight.

It is my pleasant duty to thank Dr. K. J. G. Milne for the permission to use the data of his patient (III 9).

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APPENDIX

Case Notes of Family

Case I 1.—Woman, aged 83; one of 11 children, none of whom had had anaemia, jaundice, or gallstones; one nephew had had jaundice. At 72, biliary colic and obstructive jaundice, when Hb was 68%, icterus index 110, and fragility normal. Recovery without operation. Spleen not palpable now.

II 3.—Man, aged 55. At the time of the investigations no symptoms attributable to congenital haemolytic jaundice. A year later (the fragility on fresh blood was estimated in another hospital) the spleen became just palpable.

II 4.—Woman, aged 54. Always delicate; for nine years bilious attacks without jaundice; complaints of pains in the back. One child died of hydrocephalus aged 7½ months. Spleen not palpable but enlarged radiologically.

II 5.—Woman, aged 49. "Gall bladder trouble" or "colitis" at 16, 21, and 24 years; attacks of gnawing epigastric pain at 34 years; enlarged spleen noted at 37 years; cholecystectomy at 42 years; recurrence of pain and obstructive jaundice; fragility increased, haemolysis occurring at 0.5% NaCl and above. Spleen one fingerbreadth below costal margin.

III 9.—Man, aged 20. At age 18 albuminuria; at 19 football accident produced pigmented scar. Brown patches on anterior aspect of both legs. Liver and spleen two fingerbreadths below costal margin; W.B.C. 15–25,000 per c.mm., platelets 5–800,000 per c.mm. Marrow culture showed hyperplasia especially of erythroid series; leuco-erythrogenetic ratio 0.6.

The serum bilirubin levels of these patients were 0.9, 0.9, 1.1, 2.3, and 1.4 mg.% respectively. No increased urobilinogenuria was demonstrated.

TABLES I AND II

HAEMATOLOGICAL DATA

Subject	Hb (g.%)	R.B.C.	M.C.V.	Retic. (%)	Price-Jones Curve				M.C.T.	T/D Ratio
					Mean	S.D.	Mode	Microcytes %†		
I 1	12.6	3.91	97.2	10.0	6.91	±0.57	6.5	42.5	2.60	0.38
II 1*	15.4	5.15	95.1	0.4	6.99	0.49	6.0	24.0	2.48	0.35
3*	15.0	4.9	99.0	8.0	7.23	0.48	7.0	13.0	2.41	0.33
4	13.6	4.0	91.3	12.1	6.86	0.42	7.0	39.0	2.47	0.36
5	12.6	4.1	92.0	8.8	6.94	0.45	7.0	28.0	2.43	0.35
6	13.2	4.77	88.0	0.5	7.28	0.16	7.5	12.5	2.12	0.29
III 2*	14.1	5.33	84.4	0.2	7.40	0.46	7.5	7.0	1.96	0.26
3*	15.5	5.92	84.5	0.6	6.99	0.46	7.0	28.5	2.2	0.32
4*	15.0	5.82	85.9	0.3	7.19	0.46	7.0	14.5	2.12	0.29
5*	14.5	4.6	96.7	0.8	7.25	0.49	7.5	12.0	2.35	0.32
6*	14.5	4.77	96.4	0.2	7.53	0.49	7.5	5.0	2.17	0.28
7	15.5	4.62	91.0	1.6	7.09	0.46	7.0	21.5	2.31	0.32
9	16.3	5.58	90.0	5.6	6.96	0.46	7.0	31.5	2.37	0.34
Case M	10.4	4.21	78.4	0.7	—	—	—	—	—	—
Lo	7.8	4.93	59.2	3.5	6.58	0.62	6.5	65.0	1.74	0.26
K	8.4	4.25	—	0.6	—	—	—	—	—	—
Le	9.4	4.58	76.0	—	6.82	0.64	7.0	47.5	2.08	0.31
B	6.4	3.87	63.3	3.0	6.05	0.56	6.0	91.0	2.20	0.36
Th	10.4	3.52	99.1	7.2	6.80	0.78	7.0	46.5	2.73	0.4

* 24-hour sample, smears from fresh blood.
† Smaller than 6.7 μ.

Subject	Fresh Blood			24-hour Blood		
	M.C.F.	S.D.	Minimum Osmotic Resistance	M.C.F.	S.D.	Minimum Osmotic Resistance
I 1	0.410	0.047	0.50			
II 1				0.428	0.020	0.48 tr.
3			0.48 tr.	0.527	0.070	0.70
			0.52			
4	0.420	0.042	0.48 tr.			
5	0.384	0.046	0.48 tr.	0.578	0.101	0.75
6	0.425	0.025	0.46			
III 2				0.415	0.026	0.48 tr.
3				0.443	0.032	0.50 tr.
4				0.420	0.025	0.48 tr.
5				0.420	0.025	0.48
6				0.412	0.025	0.46
7	0.398	0.026	0.48 tr.			
9	0.389	0.045	0.48 tr.	0.583	0.086	0.70
Case M	0.387	0.038	0.44			
Lo	0.422	0.069	0.50			
K	0.379	0.068	0.50			
Le	0.428	0.040	0.50			
B	0.460	0.047	0.50			
Th	0.420	0.056	0.50	0.441	0.038	0.62
D	0.408	0.023	0.48 tr.	0.429	0.027	0.48 tr.
H	0.404	0.023	0.46	0.418	0.026	0.48 tr.
Da	0.415	0.024	0.46			
P	0.400	0.020	0.46			

tr. = trace of haemolysis.

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THE ESTIMATION OF SODIUM GENTISATE IN PLASMA AND URINE

BY

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(RECEIVED FOR PUBLICATION DECEMBER 7, 1950)

The sodium salt of gentisic acid has been used in the treatment of rheumatic fever (Meyer and Ragan, 1948). It has been reported that the substance is as active therapeutically as sodium salicylate and has the advantage that it produces few, if any, toxic effects even in doses up to 18 g. per day (Camelin, Accoyer, Pellerat, Lafuma, and Coirault, 1949).

The concentrations of gentisate in plasma and urine have been measured by means of the blue colour given with ferric chloride (Camelin *et al.*, 1949) or by the determination of the optical density at 320 mμ (Consden and Stanier, 1951). In our opinion neither of these methods is suitable for routine estimations; the ferric chloride reaction is too transient for accurate work, and the measurement of the optical density at 320 mμ needs an ultra-violet spectrophotometer. We have therefore devised a photometric method of estimation based on the blue colour given by gentisate with the Folin-Ciocalteu phenol reagent in alkaline solution (Smith, 1950).

Method

Gentisic acid is quantitatively extracted from acidified plasma or urine by ethyl acetate and completely removed from the organic solvent by 1% w/v sodium bicarbonate solution at *p*H 8.5. The alkalinity of the final extraction medium is a critical factor because gentisic acid is unstable above *p*H 9.0, being converted to coloured oxidation products. A stronger alkali than 1% w/v sodium bicarbonate solution completely extracts the gentisic acid from the ethyl acetate, but also causes decomposition of the substance.

The gentisic acid in the bicarbonate extract is estimated by means of its reaction with an aqueous solution of the Folin-Ciocalteu phenol reagent, which is readily available in most clinical laboratories. A blue colour having an absorption maximum at 660 mμ develops on the addition of sodium hydroxide; the colour intensity reaches a maximum after one minute but begins to fade after 20 minutes and is reduced to 90% of the maximum intensity after two hours (Fig. 1). The absorption density of the coloured solution was found to be proportional to the concentration of sodium gentisate up to 12 mg./100 ml. in the plasma and diluted urine, and the method gave a recovery of 96% of added sodium gentisate from plasma or urine. A calibration curve (Fig. 2) may

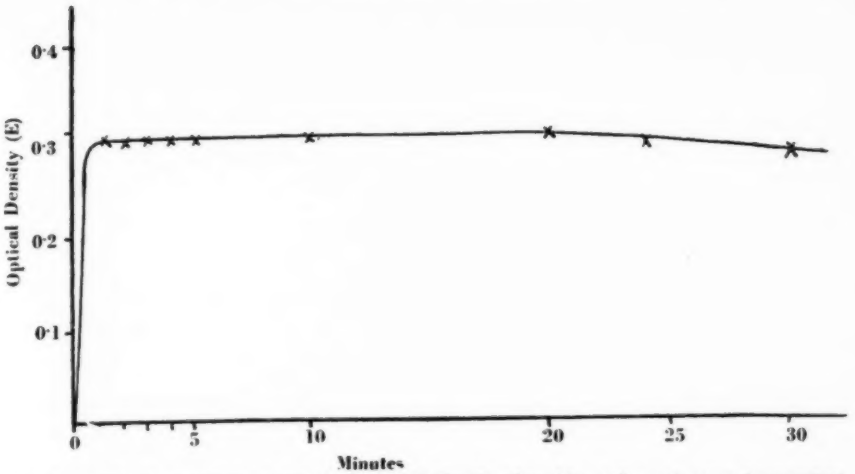

FIG. 1.—Rate of development and fading of Folin-Ciocalteu blue colour at room temperature.

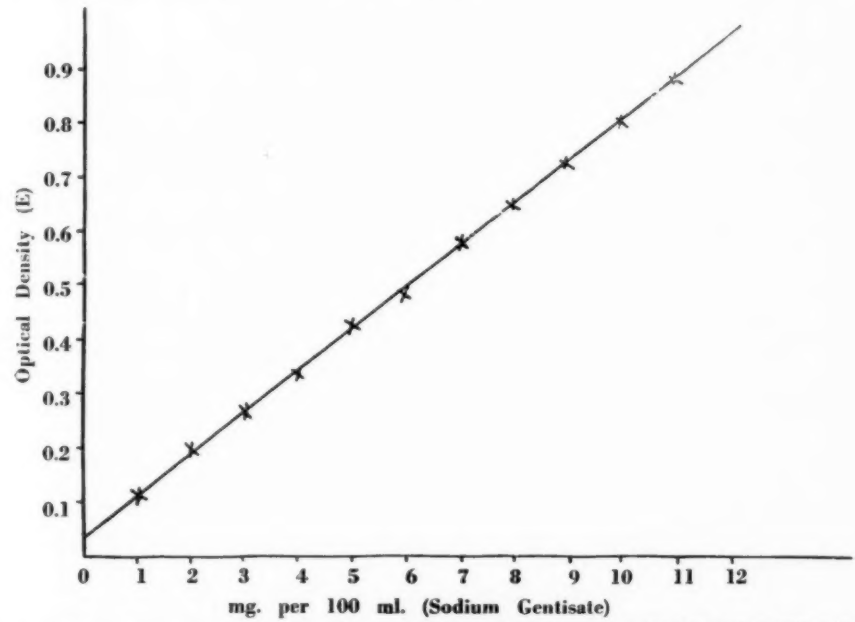

FIG. 2.—Calibration curve of sodium gentisate in distilled water, using the blue colour given by the Folin-Ciocalteu reagent in alkaline solution. The colour intensities were measured in a Hilger Spekker absorptiometer using an Ilford spectrum red filter No. 608.

be constructed from a series of solutions of sodium gentisate in distilled water, ranging in concentration from 0 to 12 mg./100 ml.

The method of estimation is as follows. To 2 ml. of plasma or 2 ml. of diluted urine (usually 1 to 100 with distilled water) 0.5 ml. of 6N.HCl is added, followed by 10 ml. of ethyl acetate. If concentrations greater than 12 mg./100 ml. are to be measured then 0.5 or 1 ml. of plasma or diluted urine are made up to 2 ml. with distilled water before the addition of the HCl and ethyl acetate. The mixture is shaken for three minutes and centrifuged for five minutes ; 5 ml. of the ethyl acetate layer are removed and added to 5 ml. of 1% w/v $NaHCO_3$ solution. The mixture is shaken and centrifuged for five minutes ; 4 ml. of the bicarbonate layer are removed and 1 ml. of Folin-Ciocalteu reagent (diluted 1 to 3 with distilled water) is added and mixed. After the addition of 1 ml. of 1.5 N.NaOH the solution is allowed to stand for five minutes and the absorption density measured against distilled water in a photo-electric absorptiometer using a 1-cm. cell and a filter transmitting maximally above 660 mμ. We have used a Hilger Spekker photo-electric absorptiometer and an Ilford spectrum red filter No. 608.

Samples of plasma and urine from patients not receiving gentisate or salicylate therapy gave negligible blank values with the procedure described above.

Results

Two women, Case 1, aged 16 years (weight 45 kg.), convalescent from a unilateral pleural effusion, and Case 2, aged 18 years (weight 58 kg.), with an artificial pneumothorax, both confined to bed, were given 12 g. of sodium gentisate per day. Four tablets (0.25 g.) were given every two hours from 6 a.m. to 8 p.m. and 16 (0.25 g.) tablets were given at 10 p.m. ; there was no restriction of fluid intake. Blood samples were collected by venepuncture at 9.40 a.m. each day and 24-hour urines were collected from 6 a.m. to 6 a.m. The sodium gentisate was given for seven days, and the plasma concentrations and urinary excretion for the two subjects are given in Table I. A representative sample of the tablets was analysed for its content of sodium gentisate by Smith's (1950) method, and the calculated total intake of the substance in each case was 80.64 g.

TABLE I

PLASMA CONCENTRATIONS AND URINARY EXCRETION IN TWO SUBJECTS

Date	Case 1			Case 2		
	Plasma (mg./100 ml.)	Urine		Plasma (mg./100 ml.)	Urine	
		24-hr. vol. (ml.)	g./24 hr.		24-hr. vol. (ml.)	g./24 hr.
October 11, 1950	0	2,140	4.708	0	2,120	4.876
,, 12	7.55	2,900	10.585	5.80	2,650	9.275
,, 13	5.50	2,430	7.655	3.82	2,820	8.320
,, 14	7.30	2,485	9.07	4.70	2,420	6.897
,, 15	4.80	3,580	16.29	3.30	2,140	12.733
,, 16	5.60	2,710	10.84	4.90	2,400	10.44
,, 17	4.55	2,540	6.223	6.15	2,400	9.852
,, 18	4.92	2,175	7.043	4.85	2,480	9.672
,, 19	0	2,540	0.508	0.65	1,910	0
,, 20	0	2,100	0	0	1,900	0
	Total excretion in urine 72.93 g. Total dosage .. .. 80.64 g. % recovery 90.4%			Total excretion in urine 72.07 g. Total dosage .. .. 80.64 g. % recovery 89.3%		

A single dose of 20 tablets (equivalent to 4.80 g. of sodium gentisate) was taken by a healthy man (weight 67 kg.), and the plasma concentrations and urinary excretion of the drug followed over a period of 24 hours (Fig. 3).

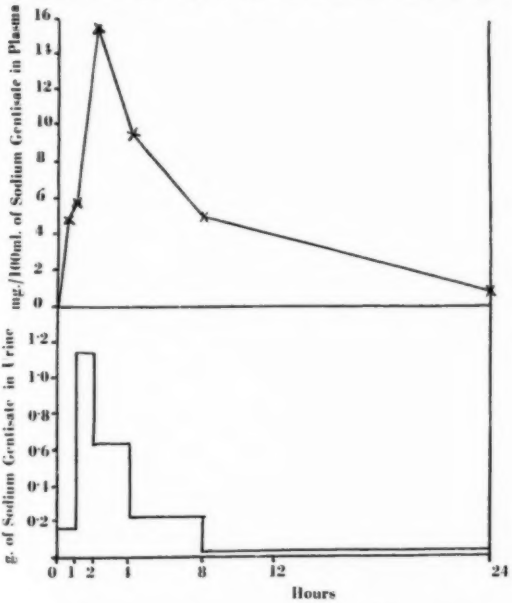

FIG. 3.—Plasma concentrations and urinary excretion over 24 hours after a single dose of 5 g. of sodium gentisate.

Discussion

These results demonstrate that about 90% of administered gentisate was recoverable from the urine, that it was rapidly excreted, and that measurable plasma concentrations of 4 to 8 mg./100 ml. were maintained in the two subjects receiving 12 g. of gentisate per day. The maximum plasma concentration (15.5 mg./100 ml.) after a single dose of 5 g. in a healthy male appeared to be reached after two hours, and 80% of the ingested dose was excreted in the urine within eight hours. These findings are not in agreement with those of Meyer and Ragan (1948), who reported that only about one-quarter of administered gentisate was recoverable from urine and that none could be detected in the blood; the method of estimation used by these authors was not given. Camelin *et al.* (1949) reported plasma levels of 7.5 to 10 mg./100 ml. in patients receiving 12 g. of gentisic acid per day in 1 g. doses every two hours, and during the course of the present work a preliminary communication by Consden and Stanier (1951) claimed that plasma levels of between 20 and 30 mg./100 ml. could be maintained by sufficiently high dosages and that about 90% of administered gentisate could be recovered from the urine.

It is realized that the results given above are limited in scope and that many factors such as body weight, fluid intake, urine *p*H and magnitude, and timing of doses may be of importance in determining the plasma concentration of the drug in any given patient.

Summary

A method of estimating the concentration of sodium gentisate in plasma and urine is described.

In two subjects receiving 12 g. per day of the substance for seven days, plasma concentrations of 4 to 8 mg./100 ml. were maintained, and about 90% of the administered gentisate was recovered in the urine. When a single dose of 5 g. was given to a healthy male the maximum plasma concentration (15.5 mg./100 ml.) appeared two hours after the dose, and 80% of the drug was recovered in the urine within eight hours.

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POTASSIUM LEVELS IN THE CEREBROSPINAL FLUID AFTER DEATH

BY

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It is well known that diffusion from the red cells makes estimation of the serum potassium after death useless as a measure of its level during life. It was thought that in the cerebrospinal fluid, whose contact with blood is relatively slight, the level might rise slowly enough for its measurement to be of value. A single estimation was enough to show that this idea was false, and that potassium diffuses rapidly into the C.S.F. after death. No record of this phenomenon could be found in the literature, and some further study was thought to be indicated. The importance of potassium in nervous tissue (Cowan, 1934; Hodgkin and Huxley, 1947; Hoagland and Stone, 1948) and in the C.S.F. itself (Watchorn and McCance, 1933; Greenfield, 1936; Helmsworth, 1947) needs no emphasis. If the rate at which the potassium level rises proved regular enough, it seemed possible that the method might have some medico-legal importance as a means of estimating the time since death.

Methods

Fifty-eight samples of cisternal cerebrospinal fluid were obtained from 46 bodies, selected at random from those coming to the hospital mortuary, excluding those with meningeal disease or anything likely to disturb the serum potassium level. About 2 ml. of C.S.F. were removed on each occasion, using the ordinary technique of cisternal puncture with a lumbar puncture needle. Samples which were slightly contaminated with blood were centrifuged, and no further samples were taken from a case once contamination had occurred. A few samples with heavy blood contamination gave unduly low readings. Multiple estimations were done in eight cases (Table I), but no more than four in any case, since it was thought that removal of more than a few millilitres of fluid might unduly change conditions in the cisterna.

Potassium levels were determined with the flame photometer, using the method described by Domingo and Klyne (1949) for serum potassium.

Results

The results are presented in Fig. 1. If potassium concentration is plotted against the logarithm of time, a very fair approximation to a straight line is produced. Assuming a linear relationship, the regression line* is:

$$K = 48.56 + 61.45 \log_{10} t$$

* The regression may be taken for the present purpose as the line which best represents one variable in terms of the other, where one of them is more liable to error than the other. Its calculation involves the determination of the line for which the sum of the squares of the deviation of the individual values from the line is least (Fisher, 1946).

TABLE I

MULTIPLE ESTIMATIONS OF POTASSIUM LEVELS IN EIGHT CASES

Case No.	Time after Death (hrs. and mins.)	Potassium Level (mg./100 ml.)
3	8.15 55.15	146 174
5	3.15 4.15	76 80
6	8.30 10.30	100 108
8	1.30 5.45 24.45	56 100 146
34	2.30 6.30 8.0 25.0	66 81 117 136
40	3.0 6.15	52 88
44	9.0 14.45	113 121
48	3.45 5.45 7.30	80 105 144

where K is the C.S.F. potassium in mg./100 ml. and t is the time in hours since death. The slope of this line is highly significant ($P < 0.001$): the standard error of the deviations of the K values from the line is ± 19.4 mg.

The degree of scatter of the results is unfortunately such that the time since death cannot be estimated with any accuracy from the potassium level. Thus, for example, a reading of 100 mg./100 ml. might be obtained between four and 18 hours after death, or occasionally outside these limits. Under the more varied environmental conditions of medico-legal work it is likely that the scatter would be even greater.

Summary

The C.S.F. potassium level rises rapidly after death.

Fifty-eight samples were obtained from 46 bodies one and a half to 70 hours after death, and the potassium level estimated with the flame photometer. The concentration increases during this period in proportion to the logarithm of the time since death.

Our thanks are due to Miss Audrey Rogers for most of the analyses, and to Dr. D. A. Mitchison for the statistical calculations.

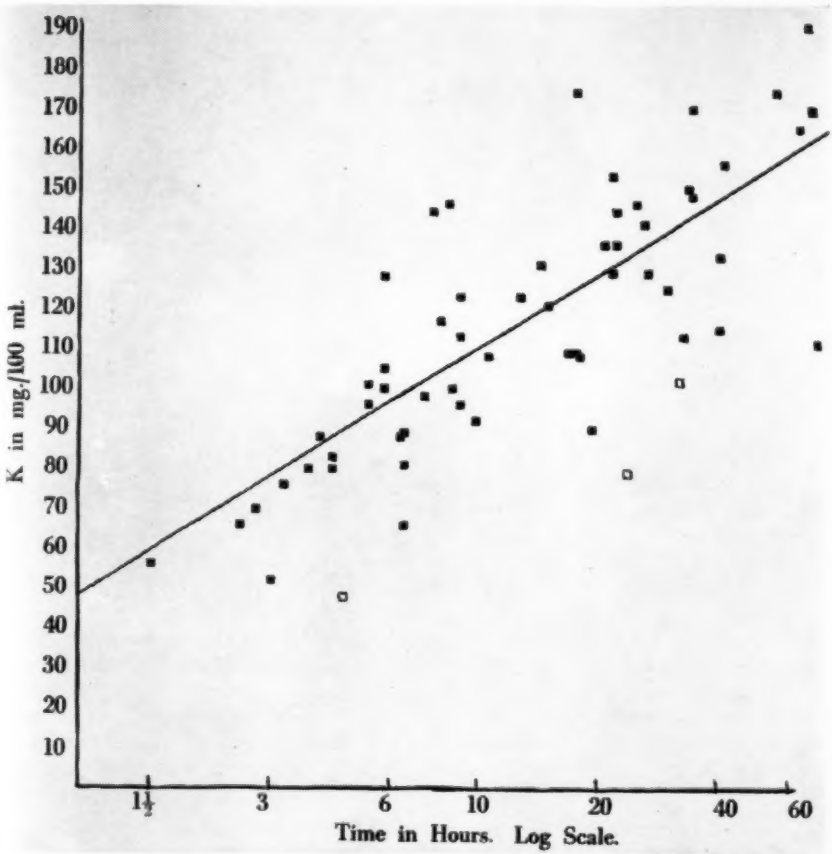

FIG. 1.—The potassium values are plotted against the log of the time (black squares). The open squares represent the values obtained with three specimens which were heavily contaminated with blood. The solid line is the regression line calculated by Dr. Mitchison.

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TECHNICAL METHODS

AN IMPROVED MICROSCOPE LAMP

BY

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The new inclined binocular microscopes when used with high-power objectives require much light to illuminate adequately the field of view. At least three times the light is necessary as for a similar optical combination on a monocular instrument. The ordinary opal bulbs even when over-run do not provide sufficient illumination, and of course with them there is no possibility of employing a light filter when using the highest powers.

There is on the market a number of high-intensity lamps using a 6-volt 5-amp. or 8-volt 6-amp. short spiral filament bulb. These have several disadvantages. The light source is so small that when using the Köhler system of illumination the back lens of the objective will not be filled unless there is a short focus lens condenser, which means that its effective diameter is small. Moreover, these lamps are small, the concentration has to be accurate, and, as the bulb has to be run almost at its full capacity, the lamp-house gets very hot. When a new bulb has to be used the centration must again be adjusted, and it may take some time to set up the whole lamp before the best working conditions are obtained. Also, on the existing lamps the filter holder is not convenient, particularly when rapid changes from one filter to another are required. These disadvantages are overcome in the design to be described.

The bulb employed is of the projection type made by Siemens (No. A 1/4), and is known as a " solid source." It has four horizontal spiral filaments arranged closely one above the other in the vertical plane, and in effect gives a solid source of light about 5 × 6 mm., which is considerably larger than the area of the low-wattage filaments in existing lamps. The bulb has a prefocus cap so that centration is done on the lamp by the maker and replacement bulbs will give the same accuracy of centration.

The body consists of an aluminium casting with lens holder and a pressed metal back. The condensing lens, specially computed for the purpose, has a focal length of 50 mm. and consists of two convex lenses. It works in a sliding mount and is focused by a convenient handle. The iris diaphragm has an aperture of 50 mm. and a long handle for easy manipulation. The filter holder is a double sliding type similar to the slide carrier on a lantern slide projector. It accommodates two 2 × 2 in. Wratten filters, which are moved into position by a touch of the finger.

The base is a hollow iron casting with four rubber feet, to which the body is hinged allowing a range of vertical movement. The body can be clamped rigidly in the required position.

Where a more diffuse source of illumination is required, as in low-power work, a finely etched ground glass screen can be brought into position immediately in front of the lamp bulb by raising the knob shown on the top right of the illustration (Fig. 1).

The full output of the bulb is 250 watts at 12 volts, and this is much too powerful for ordinary use, so the lamp is run from a 6-volt 16-amp. transformer and the intensity of light controlled by a 300-ohm sliding resistance of 0.75 amp. capacity, fitted with a switch. The resistance is connected in series with the primary of the transformer, and it is absolutely necessary for the proper working of the lamp. With the full amount of resistance, the lamp works at about 9 amps., at 4 volts giving an output of about 40 watts, which is sufficient for the 16-mm. and 4-mm. objectives on an inclined binocular microscope with 8 × eyepieces. Increased illumination for the higher power objectives is obtained by moving the slide of the resistance.

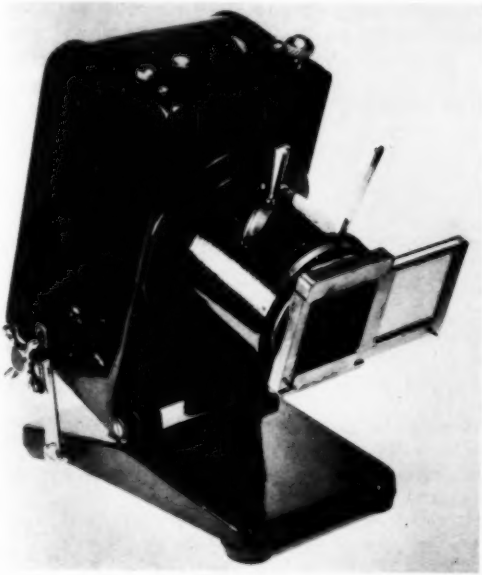

FIG 1.

As the lamp is working at much below its rated wattage, the light is more yellow than with the ordinary bulbs, and a blue filter such as is provided with the microscope, or a Wratten No. 78A filter, must be used.

The lamp is supplied with a 15-amp. domino connector so that it can readily be connected to or detached from the transformer.

The transformer itself can conveniently be mounted under the back of the bench, and the sliding resistance screwed either to a bench support or at the side of a nest of drawers or cupboard.

Method of Operating the Lamp

The Köhler method of illumination is recommended, in which the image of the filament of the bulb is focused by the lamp condenser on the iris diaphragm of the substage condenser of the microscope. The front lens of the lamp condenser will now act as the source of illumination, and it is focused by the substage condenser in the plane of the preparation to be examined, using the lamp iris diaphragm as a guide for this purpose.

The first step is to see that the substage condenser of the microscope is fully racked up, and that the piece of blue glass supplied with the microscope is in the ring under the condenser.

The microscope and lamp are set up so that the distance of the beam of light from the iris diaphragm of the lamp, by way of the mirror to the iris diaphragm of the condenser, is about 10 in.

The lamp is switched on, seeing that the lamp iris is fully open, and the resistance decreased so that there is a bright beam of light shining on the mirror. The lamp is adjusted

by altering its vertical tilt and by moving the base so that the beam of light is in the centre of the mirror.

The lamp is focused so that an image of the filament is formed on the substage iris of the microscope. In practice it will be found that this is so when the handle of the lamp condenser is moved to its forward limit. Once the correct setting is found, the lamp condenser needs no further adjustment.

The resistance is pushed back so that the light is as dim as possible, a preparation is placed on the microscope stage and focused with the low-power (16-mm. or $\frac{2}{3}$-in.) objective.

The iris diaphragm of the lamp is closed and the mirror adjusted so that the image of the lamp iris is in the centre of the field.

The microscope condenser is focused up or down slightly until the image of the lamp iris is sharp.

The lamp iris is opened so that the whole of the field is illuminated. It will be found that there is a bright and evenly illuminated field with the details of the preparation clear and distinct.

In practice these adjustments can be made in a few seconds.

To obtain the maximum definition, the lamp iris should be closed and focused in the field with each objective. This will be possible with the 4-mm. ($\frac{1}{6}$-in.) and 3·5-mm. ($\frac{1}{7}$-in.) oil-immersion objective, but not with the 2-mm.($\frac{1}{12}$-in.) objective. For routine work, however, this is not necessary.

When the ground glass diffusing screen is required, and it is very useful for low-power histological examinations, raise the knob on top of the lamp to bring it into position. The illumination will now of course be less. The resistance is moved until the correct amount of light is required.

For higher-power work with the 3·5-mm. ($\frac{1}{7}$-in.) and 2-mm.($\frac{1}{12}$-in.) oil-immersion objectives the ground glass screen should not be used. In all cases the degree of illumination is varied as required by means of the sliding resistance. It is surprising how often this will be necessary, and the fine control of the light obtained with it will be appreciated.

It is important to note (*a*) that before switching on the lamp the full resistance is in and then the illumination increased as required, and (*b*) before switching off the lamp, dim the bulb to its full amount. If these precautions are observed, the life of the bulb will be almost indefinite. There should be no blackening of the glass and no need of replacement for many years.

As the lamp is considerably under-run, the lamp-house only gets slightly warm, and even after several hours' use the hand can be placed on the lamp-housing without discomfort.

The lamp is suitable for dark field illumination, and more than sufficient light can be obtained for this purpose. If for special purposes the illumination obtained with the 6-volt transformer is not sufficient, an 8- or 9-volt transformer to take 18 amps. and tapped at 6 volts should be used. The 6-volt tapping is used for ordinary work and the higher voltage output only for special purposes.

The lamp also gives excellent illumination with phase contrast technique. It can be used with the Köhler system as already described, or, by focusing the lamp condenser back towards the bulb, parallel light can be used. In the latter case the ground glass screen should be placed in position and the lamp run a little more brightly.

The sliding filter holder is very useful when a filter is required from time to time. The filter can be kept always in position and placed in the beam of light by a touch of the finger, and the necessary increase of light made with the resistance.

This lamp and filter holder were designed not only for general microscopy but also for the following simple but efficient method of examining stained material for tubercle bacilli.

Method for Tubercle Bacilli Examination

The microscope should be fitted with a 3·5-mm. (⅛-in.) fluorite oil-immersion objective. n the filter holder are placed a Wratten No. 45 (blue-green) filter, and a Wratten neutral lter No. 96, of 10% transmission.

The preparation (smear or section) is made in the usual way, and stained by the standard iehl-Neelsen method using dilute malachite green as the counterstain. It is important that ie counterstain is not too deep.

The microscope is set up as described above, with the neutral filter in position. The ecimen is focused with the 3·5-mm. objective and the microscope and lamp adjusted to btain optimum illumination and definition. The blue-green filter is moved into position, id the background of cells will then almost disappear leaving ghosts of cells which are fficient for focusing. Tubercle bacilli in the field appear black. When searching for tubercle acilli the blue-green filter is used and the slide can rapidly and systematically be examined. s soon as a black bacillus-like object is seen, the neutral filter is moved into position, when ie slide assumes its normal colour and any red-stained bacilli observed.

A black-coloured bacillus coming into the blue-green field is instantly recognized and rified as a tubercle bacillus by moving the screen.

If further magnification is required for a doubtful object the 2-mm. objective is swung to position. The examination is then further resumed with the 3·5-mm. objective and blue-een filter. The search is rendered easy on account of the large, flat field, great depth of cus, and excellent definition of the 3·5-mm. fluorite lens.

This procedure enables a much larger area of the specimen to be examined without tigue.

I consider that this method is superior to fluorescence microscopy for detecting ubercle bacilli.

The only additions required to the lamp are the two filters, and most new binocular icroscopes are now supplied with a 3·5-mm. fluorite oil-immersion objective.

The examination can be carried out by any technician, and it is of particular value in doubtful clinical cases, as the recognition of a stained tubercle bacillus is of true diagnostic significance whereas a fluorescent bacterium is not.

This lamp is made by R. R. Beard, Ltd., 10, Trafalgar Avenue, Old Kent Road, London, S.E.15.

J. clin. Path. (1951), 4, 238.

A USEFUL COVER-SLIP CONTAINER

BY

J. C. MONCKTON

From the Southern Group Laboratory, Park Hospital, London

(RECEIVED FOR PUBLICATION NOVEMBER 16, 1950)

Most laboratory workers have experienced occasional difficulty in removing a cover-slip from its box to the slide. A container has been devised which reduces such mishaps to a minimum.

The container consists of a "perspex" box, with a central horizontal slot in the front and in the back. Through this passes a glass microscope slide with a cover-slip ($\frac{7}{8}$ in. sq. No. 1) fixed to the upper surface. Inside the box, resting on the slide, is a supply of clean cover-slips, and one is delivered through the front slot each time the slide is pushed backward and forwards.

The method of working is seen in the diagram (Fig. 1*a*), which shows the slide in the three positions required for its operation. The slide and cover-slip are shown as thick black horizontal lines. The thickness of the cover-slips is exaggerated to show the working more clearly.

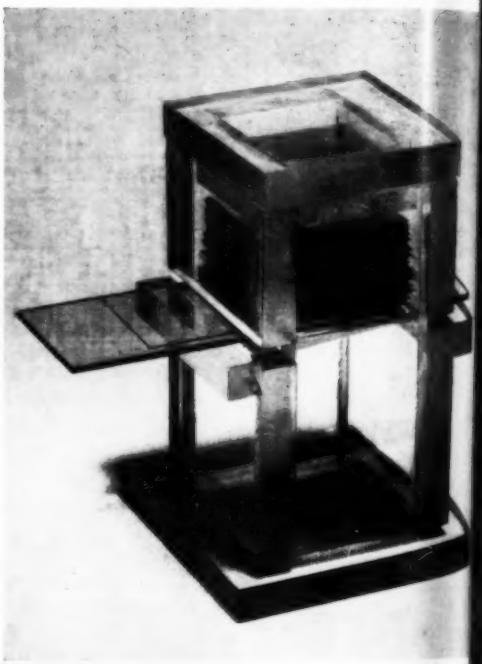

FIG. 1

Construction

A cover-slip is first mounted on a microscope slide with one edge $\frac{5}{8}$ in. from the end of the slide. Use the smallest possible amount of DPX or Canada balsam to avoid seepage beyond the edges of the cover-slip. Place in an incubator for 48 hours to dry with a small weight on the cover-slip.

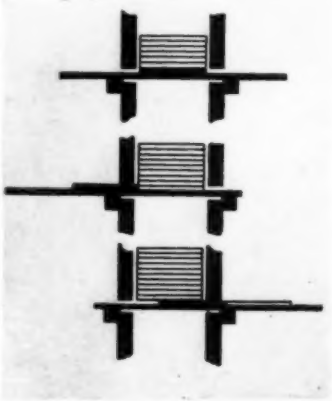

FIG. 1*a*

The box is made from " perspex " sheet 7/32 in. thick. It is cut to the sizes required and the edges are smoothed.

The sizes of the sections are as follows:

Sides	$2\frac{1}{2}$ in. × 1 in. (2 pieces)
Front and back (above slot)	$1\frac{1}{2}$ in. × $\frac{7}{8}$ in. (2 pieces)
Front and back (below slot)	$1\frac{1}{2}$ in. × $1\frac{1}{2}$ in. (2 pieces)
Base	2 in. × 2 in.
Lid (topside)	$1\frac{1}{2}$ in. × $1\frac{1}{2}$ in.
Lid (underside)	1 in. × 1 in.
Strips	$1\frac{1}{2}$ in. × $\frac{1}{4}$ in. (2 pieces)

The parts are assembled to form the box with a central slot $\frac{1}{8}$ in. wide in the front and back as shown in Fig. 1*b*.

We used a little " perspex " dissolved in chloroform as cement. The removable lid has the smaller piece fixed to its underside to keep it in place.

The two small strips immediately below the slots are fitted last. Their purpose is to correct the width of the slots to the thickness of the moving slide and cover-slip. Their position is determined by placing the selected slide through the box with the affixed cover-slip emerging from each slot in turn and cementing a strip to the front and back of the box below the slide, so as to allow just enough room for the slide and cover-slip.

The container can be fixed to the bench with two small screws through the base if desired. This will enable one to manipulate the slide with one hand.

J. clin. Path. (1951), **4**, 240.

A CENTRIFUGAL GRADOCOL FILTER

BY

S. D. ELEK AND G. R. F. HILSON

From the Department of Bacteriology, St. George's Hospital Medical School, London

(RECEIVED FOR PUBLICATION SEPTEMBER 15, 1950)

Details of a centrifugal Seitz filter for sterilizing small quantities of fluid were reported some months ago in this journal (Hilson and Elek, 1950). It was then considered that a similar filter, fitted with a gradocol membrane (Elford, 1931) for virus or bacteriophage work where an asbestos pad is not suitable, might be useful. Moreover, a suggestion has been made (Latner and Slack, 1950) that such a device could also be used for the ultrafiltration of chemical mixtures. For these purposes a different design is necessary. The necessary requirements of such an apparatus are that it should be convenient for the handling of small quantities, since often the amount of available infectious material is limited. In addition it is essential that the apparatus should be safe in use, should not cause splashing, and should not leak. Since a wide range of pressures is needed when using gradocol membranes of different porosities both positive-pressure and suction types of apparatus are commonly employed. With centrifugal force, however, the whole range needed can easily be achieved.

The filter described here (Fig. 1) is suitable for use in standard laboratory centrifuges, in certain instances an adaptor collar being required. The capacity of the filter is about 4.5 ml. The upper part or cup has a flange at its upper end to fit the centrifuge collar, a pair of facets for a spanner, and at its lower end a thread on to which the lower part of the filter is screwed. The cup is closed with a screw-cap through which fluids may be introduced with a fine pipette ; its lower end forms a socket to receive a rubber washer, the gradocol disk, and its supporting perforated plate. Below the plate there is another rubber washer, and finally a pressure ring enters the socket and bears, through the washer, on the lower surface of the plate. This ring is prevented from rotation, relative to the socket and its contents, by means of a lateral lug which engages a slit in the side of the socket. The pressure ring has a funnel extension to direct the filtrate down the central axis, and it has a smooth bearing surface on its under side. The lower portion of the apparatus consists of a holder, having at its upper end a deep internal thread, a similar pair of spanner facets, and a stage with a smooth upper surface, which forms a rotating bearing with the pressure ring. Below this stage the holder contains a glass tube to receive the filtrate, and is closed at its lower end by a screw-cap. All those parts of the filter which will come in contact with fluid are made of stainless steel.

For use, the holder is loaded with a glass tube and, together with the other metal parts, is sterilized by dry heat. The rubber washers are autoclaved, and filter disks of the right diameter are steam sterilized. The parts are then assembled, preferably under ultra-violet light. Alternatively, there seems to be little objection to assembling the filter loosely, with the membrane in position, and sterilizing by intermittent steaming. The filter is screwed up tight with spanners. As this is done, the stage bears on the pressure ring and drives it up the socket, so compressing the edge of the filter disk between the system of washers and perforated plate, and rendering the joints water-tight. The filters are centrifuged in pairs, and the required filtration pressure is obtained by adjusting the centrifuge speed according to a nomogram which correlates speed, distance of the disk from the axis, and the depth of fluid in the cup with the pressure developed (Fig. 2).

Summary

A centrifugal filter for use with gradocol membranes is described.

The filter is available in the size described and in a larger capacity model from Messrs. M.S.E., Ltd., 14, Spenser Street, London, S.W.1.

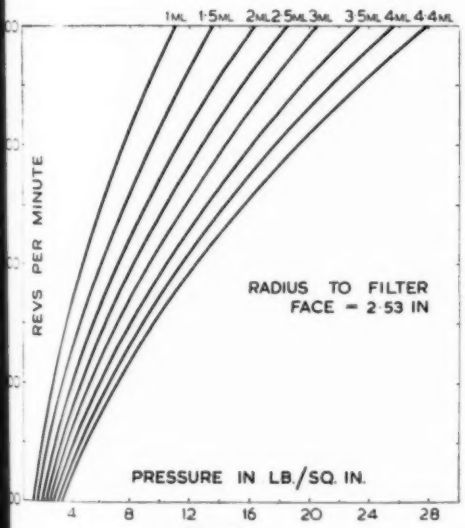

FIG. 2

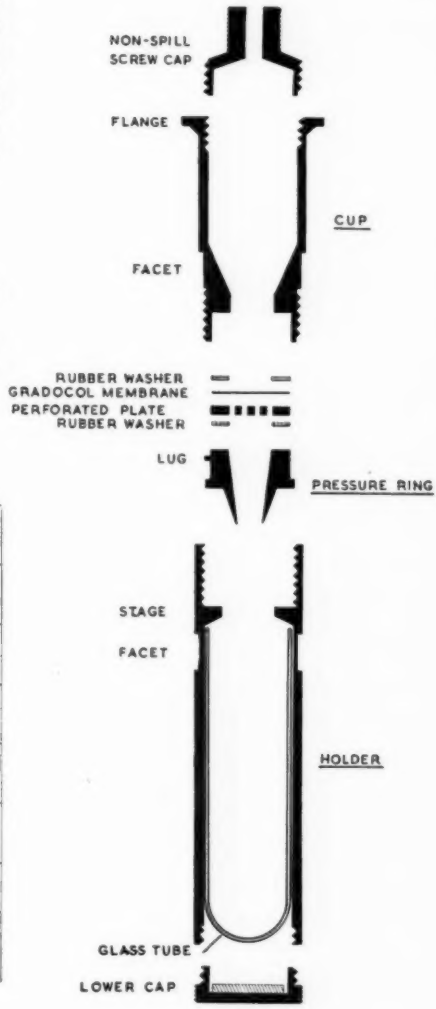

FIG. 1

We wish to express our sincere thanks to Dr. W. J. Elford, F.R.S., of the Medical Research Council, for his kindness in testing the prototypes for us and for his valuable advice.

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J. clin. Path. (1951), **4**, 243.

ABSTRACTS

This section of the JOURNAL *is published in collaboration with the two abstracting journals, Abstracts of World Medicine, and Abstracts of World Surgery, Obstetrics and Gynaecology, published by the British Medical Association. In this* JOURNAL *some of the more important articles on subjects of interest to clinical pathologists are selected for abstract, and these are classified into four sections: bacteriology; biochemistry; haematology; and morbid anatomy and histology.*

BACTERIOLOGY

Synthetic and Fermentation Type Chloramphenicol (Chloromycetin) in Typhoid Fever: Prevention of Relapses by Adequate Treatment. SMADEL, J. E., BAILEY, C. A., and LEWTHWAITE, R. (1950). *Ann. intern. Med.*, **33**, 1.

A continuation of the studies on the use of chloramphenicol ("chloromycetin") in the treatment of patients with typhoid fever indicates that the synthetic form of the drug is as efficacious as the natural antibiotic obtained by the fermentation process from *Streptomyces venezuelae*. The same total amounts of either type drug are equally effective when given in divided doses at 2- to 6-hour intervals or in larger doses once or twice daily.

There was a definite relationship between the duration of chloramphenicol treatment and the occurrence of relapses in typhoid fever. Slightly more than half of the patients who were treated for 8 days or less had a recrudescence of the disease which began about 10 days after treatment was stopped. No relapses occurred in the groups of patients treated for longer periods of time. The present data suggest that a 14-day period of treatment is sufficient to prevent relapses. In spite of the dramatic therapeutic effectiveness in patients with typhoid fever, serious complications such as intestinal haemorrhage and perforation may be expected in treated patients since the stage is generally set for such developments before therapy is instituted and time is required for the healing of the typhoidal lesion of the intestine. In the present group of 23 patients, 2 had haemorrhage sufficiently severe to produce shock. Two other patients suffered intestinal perforation; the course in one of these was further complicated by severe haemorrhage and the disease terminated fatally. Neither of the patients with perforation was given surgical treatment; chloramphenicol therapy controlled or suppressed the usual signs of generalized peritonitis.

On the basis of the present observations, it would appear that the adequate treatment of typhoid fever in the adult consists of an initial oral dose of 3.0 to 4.0 g. of chloramphenicol, followed by 1.5-g. oral doses given every 12 hours during the febrile period and by single daily 1.5-g. doses for 7 days; thereafter the dose may be reduced to a single 1.0-g. dose and continued until the 14th day of antibiotic therapy, after which the drug may be discontinued. Particular attention should be given to the recognition of intestinal perforation in treated patients, since the classical signs of this development with the ensuing generalized peritonitis may be partially masked by the antibacterial effect of chloramphenicol.—[Authors' summary.]

***In vitro* Activity, Absorption and Excretion of Chloromycetin.** TUNEVALL, G., and FRISK, A. R. (1950). *Scand. J. clin. Lab. Invest.*, **2**, 162.

Using 0.05 ml. of a 10^{-3} dilution of 18-hour cultures as inocula, the authors compared the effects of chloramphenicol (in brain-heart infusion) *in vitro* with those of

aureomycin (in thioglycollate broth). Chloramphenicol was assayed in the brain-heart infusion medium, with a strain of *Klebsiella pneumoniae* (sensitive to 0.4 μg. per ml.) as test organism. With 8 strains of Gram-positive organisms, aureomycin was some 30 to 100 times more active and with 8 strains of Gram-negative organisms (except for *Proteus vulgaris*) some 5 times more active than chloramphenicol.

Single oral doses of 1 g. chloramphenicol gave maximum levels in blood in 1 hour (10 μg. per ml.) and the antibiotic had disappeared in 24 hours. With 1 g. 8-hourly, a mean level of 4 to 10 μg. per ml. in blood was attained. Urinary excretion was also very rapid, 80% being excreted in 4 hours, and excretion was complete in 8 to 12 hours. Conjugation starts rapidly and the antibiotic is largely excreted in the urine in the inactive form. Thus chloramphenicol is absorbed and excreted more rapidly than aureomycin, and the optimum interval between oral doses should be 4 hours. *Malcolm Woodbine.*

A Case of Brill's Disease in London. HAWKSLEY, J. C., and STOKES, E. J. (1950). *Lancet*, **2**, 97.

The history of Brill's disease is given.

The present authors describe the case of a woman aged 49 who developed a febrile illness, with headache and pains in the limbs, which was treated with sulphadimidine (" sulphamezathine ") without effect. The symptoms had not changed by the 8th day of illness, when the patient was admitted to hospital. The temperature was then 102° F. (38.9° C.) and there was suffusion of the conjunctivae, but no rash was present and the spleen was not palpable. Her temperature began to fall after admission to hospital and was normal after 8 days. The patient was born in Poland and had typhus there in 1915; she migrated to Berlin in 1919, and to London in 1939. A tentative diagnosis of Brill's disease was made and investigations (blood count, blood culture, faeces culture, agglutination tests, etc.) were undertaken. All were negative except the Weil–Felix tests, in which an agglutination titre of 1 in 700 to *Proteus* OX19 antigens was obtained on the 16th day of illness, subsequently falling rapidly. Samples of serum were sent for further investigation to Felix, whose opinion was that the marked drop in the OX19 titre was to be accepted as confirming the clinical diagnosis. Specimens of serum were also sent to Washington for rickettsial agglutination and complement-fixation tests, the reactions being reported as " definitely epidemic in type and . . . consistent with a diagnosis of Brill's disease."

A Note on a Case of Brill's Disease in London. FELIX, A. (1950). *Lancet*, **2**, 99.

Apart from one case reported by Mooser and Loeffler in 1946 in Zurich, all the reported cases of Brill's disease have occurred in the coastal towns of the north-eastern United States. Although the majority of cases have been in Jewish immigrants from eastern Europe, the incidence is not limited to any racial group. It seems likely that the disease will occasionally be seen in any country whose population includes an appreciable proportion of persons previously exposed to louse-borne typhus.

The clinical diagnosis of Brill's disease is not easy, as the disease is always sporadic and the clinical course milder than in the primary attack of louse-borne typhus. In the present case the patient did not even develop a rash, which is the most helpful of the few diagnostic signs. Laboratory tests are indispensable for diagnosis, and, although agglutination and complement-fixation tests became available recently and are valuable in differentiating the varieties of typhus, the case under review shows clearly that it was possible to confirm the diagnosis by the use of the *Proteus* OX19 reaction; incidentally, it became positive at an earlier stage than the rickettsial agglutination test.

It is important, however, that the Weil–Felix test be standardized by international agreement. The customary use of Dreyer's technique is unsatisfactory because of the heat-lability of O agglutinins. The proper technique entails the use of round-bottomed

ubes and incubation at 37° C. (Felix, *Trans. R. Soc. trop. Med. Hyg.*, 1944, **37**, 321). How great is the need for standardization is shown by results published by Murray *et al.* (*J. Amer. med. Ass.*, 1950, **142**, 1059), who noted that only 3 out of 14 sera from patients with Brill's disease gave a diagnostic agglutination titre in the Weil–Felix test. The indications are that the technique employed was unsatisfactory, dilutions as low as 1 in 10 being used. In the London case the tests carried out in two laboratories showed a fourfold difference in titre.

From the epidemiological point of view Brill's disease is of interest as a possible source of dissemination of louse-borne typhus, and, in view of the widespread epidemics of this form of typhus during the last war and the subsequent movement of large groups of people from one country to another, the disease may come into prominence in many parts of the world.

J. V. Armstrong.

BIOCHEMISTRY

Plasma Protein Studies of 200 Cases of γ-Hyperglobulinaemia and their Clinical Application. WUHRMANN, F., WUNDERLY, C., and DE NICOLA, P. (1950). *Z. klin. Med.*, **147**, 73. Bibliography.

Electrophoretic analysis of sera in 24 cases of myelomatosis revealed a significant decrease in γ-globulin mobility compared with eight normal sera. In 23 cases of hepatic disease, tuberculosis, or malignant disease, there was no change. Electrophoretically homogeneous γ globulin was prepared in eight cases of myelomatosis, by methanol precipitation of the sera at *p*H 6.8, in the cold. Extinction coefficients, at 280 mμ. and *p*H 2 and >12, differed widely, lying above and below the normal. These results support the hypothesis of the heterogeneity of the γ globulins. In the electrophoretic patterns, the peaks of myeloma γ globulin were steeper and narrower than corresponding peaks in other diseases. The authors consider the shape of the peak to indicate the degree of heterogeneity of the γ globulins, the ratio of height to width of base (Q) decreasing with increasing heterogeneity.

Out of 800 sera analysed by electrophoresis, 200 had a raised γ-globulin content, that is, this globulin formed more than 20% of the total protein (normal = 14 to 18%). In each case, the diagnosis and the figures for protein and Q are recorded. In 150 cases the cadmium sulphate turbidity test and Weltmann calcium-chloride heat-coagulation test were performed.

In myelomatosis, γ globulin forms about 40% of the total protein, the amount of the latter being also raised. The cadmium reaction is positive and there is a marked shift to the right in the Weltmann reaction. The empirical protein reactions are strongly positive. The erythrocyte sedimentation rate is typically raised and Q is increased, this change being almost pathognomonic.

In malignant disease all three globulin fractions increase, but there is a subnormal or low value for total protein. The cadmium reaction is positive and there is a shift to the left in the Weltmann test.

In alcoholic cirrhosis of the liver the amount of γ globulin is moderately increased while those of α and β globulin are slightly raised. The cadmium reaction is positive and the Weltmann test shows a widened range of coagulation. In acute infections α globulin increases in amount as well at γ globulin. In chronic infections the results are variable. *M. Lubran.*

Serum and Urinary Potassium in Surgical Patients. SNYDER, C. D., and SNYDER, H. E. (1950). *Arch. Surg., Chicago*, **61**, 62.

In 38 consecutive surgical cases involving major operation (including 15 on the gastro-intestinal or biliary tracts) and five involving minor operation, complete analyses of the blood, urine, and gastric return were made at frequent intervals; whenever possible, the plasma potassium

concentration was determined 24 hours and 1½ hours before operation, immediately after operation, and then 24-hourly until a normal level was reached and every 48 to 72 hours thereafter during the remainder of the patient's stay in hospital. The clinical management of the patient was not altered in any way and parenteral fluids were given in accordance with the usual routine; potassium therapy was not given unless it was considered essential [presumably on biochemical as well as on clinical grounds].

A plasma potassium level below 3.8 mEq. per litre was found [at one or more readings?] in 26 cases. The levels at different intervals were extremely variable, sometimes fluctuating widely between one estimation and the next. Of 26 cases in which the immediate pre-operative and post-operative plasma potassium levels were determined, in 13 there was a rise after operation, and in 13 a fall. The lowest average reading in the 38 major surgical cases was 3.25 mEq. per litre, and the highest 4.97 mEq. per litre. [Presumably these averages refer to the complete period of study for each patient.] It is apparent, therefore, that any conclusions drawn from the average plasma potassium values in the different periods studied would have no value. Urine volume and urinary excretion of potassium also tended to vary rather widely; the urinary excretion rates for water were higher, and for potassium were lower, than those reported by other workers. The nearest approach to a set pattern of reduction in plasma potassium values associated with a rise in urinary excretion of potassium was exhibited by the six patients who had undergone cholecystectomy. Potassium therapy had been considered essential (and had been given) in seven of the 26 cases in which hypopotassaemia was found; the clinical and biochemical findings indicated that it could have been given with advantage in 11 other cases. Clinical notes with graphs are given of some typical cases, including three in which potassium therapy could be regarded as life-saving. The series included many elderly patients and a number of poor operative risks; infusion therapy could not be considered a factor in the three deaths which occurred.

In a series of cases in which plasma potassium values were determined less frequently, levels below 3.8 mEq. per litre were found in 9 of 30 patients undergoing operations on the biliary tract, and in 8 of 42 other surgical patients. In all, 100 major surgical cases were studied. It is concluded that "studies on surgical patients have clearly demonstrated the necessity for a knowledge of plasma potassium levels in the management of complicated surgical cases."

[The authors of this important paper could have been more informative. How many of the patients, for instance, showed one or more plasma potassium levels below, say, 2.3 mEq. per litre in the immediate post-operative period?]

Joseph Parness.

The Effect of Diethylstilbestrol on the Calcium, Phosphorus and Nitrogen Metabolism of Prostatic Carcinoma. SCHILLING, A., and LASZLO, D. (1950). *J. clin. Invest.*, **29**, 918.

The metabolism of a patient with diffuse osseous metastases from a carcinoma of the prostate was studied before and during four months' oestrogen therapy at the Montefiore Hospital, New York. Urinary and faecal excretion of calcium, abnormally high before, decreased during therapy, and the calcium balance changed from negative to positive. The serum acid-phosphatase level fell markedly; the serum alkaline-phosphatase level rose slightly. Nitrogen and phosphorus metabolism was little affected. The theoretical phosphorus balance, based on a nitrogen/phosphorus ratio of 15:1 and a calcium/phosphorus ratio of 2.2:1, was calculated. Throughout the period there was retention of phosphorus, unaccountable theoretically, which was less after treatment that before. *P. Mestitz.*

Hypercalcemia in Osteolytic Metastatic Cancer of the Breast. SWYER, A. J., BERGER, J. S., GORDON, H. M., and LASZLO, D. (1950). *Amer. J. Med.*, **8**, 724.

Working at the Montefiore Hospital, New York, the authors of this paper have studied a series of 71 patients with osteolytic bone metastases from cancer of the breast. In 10 of these they observed a syndrome, consisting of gastro-intestinal disturbances, mental changes, and renal insufficiency, which was associated with hypercalcaemia. In these 10 cases the serum calcium content ranged from 12.3 to 20 mg. per 100 ml., whereas the serum inorganic-phosphorus and alkaline-phosphatase levels did not consistently vary from normal. The patients frequently showed evidence of dehydration, and an elevated blood urea-nitrogen level and impaired renal clearance were found in all cases. The authors believe actual bone destruction by the tumour to be the most important factor in the development of the hypercalcaemia, but they discuss other factors, including immobilization and renal insufficiency, which they regard as contributing factors.

Treatment took the form of restriction of calcium intake and improvement of hydration by the parenteral administration of fluids, with sodium citrate in some cases, to facilitate calcium excretion. Although the ultimate prognosis was not affected, response to treatment is described as frequently dramatic, and was associated with the return of serum calcium content and blood urea level to normal.

H. A. Sissons.

Regulation of Sodium Excretion in Normal and Salt-depleted Subjects. BLACK, D. A. K., PLATT, R., and STANBURY, S. W. (1950). *Clin. Sci.*, **9**, 205.

The osmotic stability of the body fluid is maintained by keeping the serum sodium level constant. This level is kept constant by a renal excretion of sodium which effectively compensates for changes in the sodium intake and in the amount of sodium lost by sweating. It has hitherto been believed that the amount of renal excretion is influenced by serum sodium level and the glomerular filtration rate (GFR), which in turn depends on the sodium load presented to the kidney for excretion. The authors, by studying the excretion of sodium and other substances like chloride, bicarbonate, potassium, urea, sulphate, and phosphate in normal and salt-depleted subjects, were unable to confirm this hypothesis. They found that, in all subjects, increase in the sodium load which directly affects the GFR did not lead to increase in sodium excretion even after the serum sodium level and inulin clearance had been raised to normal figures or figures above normal. This means that sodium reabsorption occurs in the tubules which must, therefore, play a significant part in determining the amount of sodium excretion. The authors found that in salt-depleted subjects sodium reabsorption was not only greater but also more prolonged, and was maintained for a few days. They therefore believe that the slowness with which the readjustment to normality takes place suggests that a hormonal mechanism may be concerned, and that there may be an overproduction of adrenal cortical hormones during salt depletion. [Full experimental plan and methods are given and the results are well tabulated.]

S. Karani.

HAEMATOLOGY

The Relationship of Polycythemia Vera to Leukemia: A Critical Review. [In English.] SCHWARTZ, S. O., and EHRLICH, L. (1950). *Acta haemat., Basel.*, **4**, 129. Bibliography.

The whole literature relating to the coincidence of polycythaemia vera and leukaemia is critically reviewed, but only 30 cases out of the 83 reported in the literature are accepted by the authors as adequately proved. In 25 of these 30

cases irradiation treatment had been given for polycythaemia before leukaemia became manifest, in three others such treatment may have been given, and in another the leukaemia developed at the time of starting radiotherapy. In only one case of polycythaemia did leukaemia undoubtedly develop without any previous irradiation.

The type of leukaemia which develops in cases of polycythaemia after *x*-ray therapy may be either of the acute or of the chronic granulocytic type, whereas in all those cases recently reported of leukaemia following treatment of polycythaemia with radioactive phosphorus were of the acute granulocytic type. The authors regard the evidence of a causative relationship between irradiation and the development of leukaemia as proven.

A. Piney.

Microscopic and Histochemical Studies on the Auer Bodies in Leukemic Cells. ACKERMAN, G. A. (1950). *Blood*, **5**, 847.

Auer bodies were found in the abnormal leucocytes in 16 cases of acute monocytic leukaemia and four cases of acute myelogenous leukaemia out of a total of 185 cases of acute leukaemia occurring in the haematological clinic at Ohio State University during a period of 3½ years. Histochemical studies were made on blood specimens from seven of the cases of monocytic and three of the cases of myelogenous leukaemia, the method including the examination of supravital preparations both by direct and phase contrast microscopy. The staining properties of the Auer bodies are described. It is suggested that they are formed by the fusion of newly formed "normal" cytoplasmic granules which take place in young cells in which the granules are of a relatively acid *p*H. *P. C. Reynell.*

Myeloid Metaplasia. BLOCK, M., and JACOBSON, L. O. (1950). *J. Amer. med. Ass.*, **143**, 1390. Bibliography.

Twelve cases of myeloid hyperplasia seen within a period of 18 months are described. In two cases the condition occurred as the terminal stage of polycythaemia and in three cases as a result of carcinoma with skeletal metastases; two cases were associated with tuberculosis, one case was due to sclerosis of the bone marrow, and four appeared to be "primary." The clinical and haematological features correspond well with those already described in the literature. The bone-marrow findings were variable; only in one case was myelosclerosis generalized, but in six of them one sternal puncture failed to produce enough cells to make adequate smears. In the hyperplastic marrow the erythroblast was the dominant cell, in marked contrast to the findings in leukaemia. The importance of hepatic and splenic biopsy examination in diagnosis is stressed. Extramedullary haematopoiesis was demonstrated by these methods in every case in which they were carried out. The differential diagnosis from leukaemia is discussed in detail. [This is a lucid review of a subject which often causes difficulty.] *P. C. Reynell.*

Nine Blood-group Antibodies in a Single Serum after Multiple Transfusions. COLLINS, J. O., SANGER, R., ALLEN, F. H., and RACE, R. R. (1950). *Brit. med. J.*, **1**, 1297.

A Connecticut woman aged 40 years had two pregnancies, the first ending in toxaemia and premature stillbirth, and the second in normal delivery. At this time splenomegaly was noted, which was said by the patient to have been present for 12 years. No abnormality was found on examination of the blood on three occasions. Four years later splenectomy was performed, the spleen weighing 1,100 g., after which anaemia developed and two blood transfusions were given. During the next 12 years the patient was given a further 18 transfusions for anaemia due to osteosclerosis, and on four occasions developed a transfusion reaction. On examination of the patient's serum, nine blood-group antibodies were found, as shown in the following table:

System	Phenotype	Genotype	Antibodies in the Serum
ABO ..	O	OO	Anti-A and anti-B
MNS ..	MsMs	MsMs	Anti-N and anti-S
Rh ..		CDe/cde or CDe/cDe	Anti-E and anti-C^w
Kell ..	K-	kk	Anti-K
Lewis* ..	Le(a- b-)	?Le^{c}Le^a	Anti-Le^a and anti-Le^b
P ..	P-	pp	—
Lutheran*	Lu(a-)	Lu^{b}Lu^b	—

* See Andresen *et al.*, *Nature*, 1949, **163**, 580.

Of these antibodies, those to E, K^w, C, Le^a, and S were probably immune in origin and the remainder naturally occurring. It is surprising that among this wealth of antibodies, five of which are very rare, no anti-P was found, since the patient lacked the antigen P and she must surely have received some P+ blood.

John Murray.

Multiple Antibody Response to Repeated Transfusions. MALONE, R. H., and COWEN, J. (1950). *Brit. med. J.*, **1**, 1299.

A woman of 64 received transfusions of blood from 24 donors "compatible" in respect of A, B, O, and D blood antigens, as a result of which she developed antibodies to E, M, and P. The authors state that routine methods of cross-matching are inadequate to detect the rarer antibodies, and that special techniques should be employed in cases of repeated transfusion. They suggest that a search for rare antibodies in the sera of patients who have been repeatedly transfused with so-called compatible blood will reveal that these antibodies are of much more frequent occurrence than is generally believed.

John Murray.

Dangerous Universal Donors. II. Further Observations on *in vivo* and *in vitro* Behaviour of Isoantibodies of Immune Type Present in Group O Blood. ERVIN, D. M., CHRISTIAN, R. M., and YOUNG, L. E. (1950). *Blood*, **5**, 553.

The authors describe the occurrence of severe haemolytic reactions in three group-A (sub-group A_1) individuals on transfusion with group-O whole blood or plasma. In one case 10 ml. of a commercial preparation of soluble A and B factors had been added to 500 ml. of whole blood before transfusion. The anti-A antibodies demonstrated in the donors' serum fixed complement, acted as haemolysins, were difficult to neutralize with soluble A and B factors, and were capable of giving a positive reaction to Coombs test, while their ability to agglutinate group-A erythrocytes was enhanced by normal human serum. In other words, they had the characteristics of antibodies observed in serum from donors known to have been actively immunized against the A factor by previous transfusion of group-A erythrocytes, by transfusion of A factor, or by pregnancy. A history of such a stimulus was, however, lacking in the case of these donors. Small amounts of immune A antibody were consistently demonstrated in 12 of 100 random samples of group-O serum which, after neutralization, gave indirect positive Coombs tests with A_1 erythrocytes and agglutinated A_1 erythrocytes suspended in compatible normal human serum.

The authors conclude that there is no longer any justification for regarding group-O blood as universally safe for transfusion purposes. They stress that, while outward clinical manifestations of a haemolytic reaction may be slight, the laboratory findings may be dramatic. The addition of group-A and group-B substance is not at present sufficient to render all group-O blood safe, and they suggest that all group-O blood should be screened. Many dangerous donors would be eliminated if their serum, diluted 1 in 50, 1 in 100, or 1 in 200 with saline, were tested with a saline suspension of group-A and group-B erythrocytes. If group-O plasma which in such dilution failed to agglutinate erythrocytes of either group were neutralized with soluble A and B factors, some reduction in hazard would be achieved. Tests of neutralized serum against group-A and group-B erythrocytes suspended in the recipient's own serum would serve as an additional precaution in selected cases, especially those receiving multiple transfusions from universal donors.

Janet Vaughan.

Coagulation Defects associated with Premature Separation of the Normally Implanted Placenta. WEINER, A. E., REID, D. E., and ROBY, C. C. (1950). *Amer. J. Obstet. Gynec.*, **60**, 379.

The authors suggest that alterations in the blood coagulation mechanism may be associated with premature separation of the placenta, and investigations on three severe cases of this condition are described.

In the first case described the blood components were normal one week before separation of the placenta. Three hours after separation, and without rupture of the membranes, the fibrinogen plasma and prothrombin levels were 50% of normal and there was evidence of a circulating fibrinolysin. The blood findings were normal again three days after delivery. In the second case the plasma fibrinogen value four hours after placental separation was one-third of normal. There was a spontaneous increase 20 hours after rupture of the membranes, and a further increase due to blood transfusion at delivery. The prothrombin activity, which was 80% after the onset of symptoms, fell to 30% at delivery, but also improved with transfusion. A circulating fibrinolysin was present before artificial rupture of the membranes. In the third case the coagulation mechanism was normal four hours after placental separation. Two hours later, in spite of transfusion, fibrinogen was absent, prothrombin activity was diminished, and a circulating fibrinolysin was present. There was persistent bleeding after delivery, with no sign of clotting. A total of 2,400 mg. of fibrinogen was given intravenously in solution over a period of half an hour, and bleeding ceased. The blood fibrinogen level had risen by then to 187 mg. per 100 ml.

The authors believe that the changes in the coagulation mechanism follow rather than precede premature separation of the placenta. In 15 other patients, apparently bleeding from a partial separation of placenta, the coagulation mechanism was normal. It is felt that there is a need for a rapid test which will detect a critical reduction in blood fibrinogen concentration, and observation of the size and stability of the clot in a sample of incubated venous blood is suggested as a suitable test. Transfusion of 1,500 ml. of blood, with the addition of 2,000 to 6,000 mg. of fibrinogen, is an important part of treatment when clot stability is inadequate. *Margaret C. S. Binnie.*

Investigations on the Role of Plasma Cells as Antibody Producers. [In English.] GORMSEN, H. (1950). *Sang*, **21**, 483.

The existing evidence for the globulin-forming function of plasma cells is briefly indicated. In a study of sternal marrow from 800 patients with diseases other than multiple myelomatosis the author found that a plasma cell count of more than 5% was invariably accompanied by hyperglobulinaemia. In patients with a serum globulin level of more than 3.5 g. per 100 ml. the converse was almost always true. Earlier work showed that rabbits hyperimmunized with polyvalent pneumococcal vaccine responded with an intense generalized plasma-cell proliferation. The antibody content of tissue extracts was related to the number of plasma cells in the tissue. "Stilbamidine," although producing specific myeloma cell inclusions in patients with myelomatosis, did not apparently interfere with antibody formation in experimental animals. *M. McC. Giles.*

MORBID ANATOMY AND HISTOLOGY

Multiple Cancers: Primary in the Lung and Other Sites. CAHAN, W. G., BUTLER, F. S., WATSON, W. L., and POOL, J. L. (1950). *J. thorac. Surg.*, **20**, 335.

Among 1,493 cases of primary carcinoma of the lung observed at the Memorial Hospital, New York, over a period of 24 years, 25 cases (1.6%) of multiple primaries were encountered, two tumours being present in all but one case, in which there were three. In 7 cases the tumours had different histolo-

gical appearances (positive evidence); in 13 the tumours had similar histological appearances, but on clinical grounds it was unlikely that either was a metastasis from the other (probable evidence); and in 5 cases the lung lesion was associated with a basal-cell carcinoma of the skin. The oral cavity (7), skin (5), and larynx (4) were the most frequent sites of the second tumour. In 15 instances diagnosis of the 2 lesions was made within 6 months of each other, and in 10 cases the second lesion was diagnosed more than 6 months after the first.

Histogenesis of Papillary Cystadenoma Lymphomatosum (Warthin's Tumor) of the Parotid Salivary Gland. THOMPSON, A. S., and BRYANT, H. C. (1950). *Amer. J. Path.*, **26**, 807.

This is a well-illustrated review of the subject of adenolymphoma, the view being supported that this tumour arises from parotid ducts, and that it and "oxyphilic granular cell adenoma are related neoplasms that differ only in pattern and supporting stromal elements." Adenolymphomata fall into two histogenetic groups: (1) those which arise from parotid ducts included in lymph nodes; (2) those which arise in the parotid gland itself and are accompanied by an inflammatory aggregation of lymphoid tissue. The close proximity of the lower part of the parotid gland to the submandibular gland probably accounts for the supposed origin of some adenolymphomata from the latter. [A full bibliography adds to the value of this useful paper.] *R. A. Willis.*

Amyloid and Myeloma. DAHLIN, D. C., and DOCKERTY, M. B. (1950). *Amer. J. Path.*, **26**, 58.

Amyloid material, giving characteristic reactions with methyl violet, methyl green, van Gieson, and Congo red stains, was found in 14 out of 66 personally studied cases of plasma-cell tumours. In 2 of the cases intracytoplasmic inclusions, staining weakly for amyloid, were present in the larger tumour cells. It is suggested that the presence of amyloid within a tumour of debatable nature is almost diagnostic of myeloma; it is also thought that plasma cells produce amyloid or some precursor of the latter. Necropsy findings in 2 cases of myelomatosis are also described; in one there were widespread amyloid deposits, in the other local deposits only.

A. Wynn Williams.

Malignant Blood Vessel Tumors. A Report on 56 Cases of Angiosarcoma and Kaposi's Sarcoma. MCCARTHY, W. D., and PACK, G. T. (1950). *Surg. Gynec. Obstet.*, **91**, 465.

A comparative survey of 20 cases of angiosarcoma and 36 cases of Kaposi's sarcoma is presented. An attempt has been made to clarify the confusion existing in the study of malignant blood-vessel tumours. Definite predilections in race, sex, and anatomical onset were noted in Kaposi's sarcoma. The condition is bizarre or fulminating in females. Similar predilections were noted in the patients with angiosarcoma.

Trauma appears to be directly involved in the development of angiosarcoma in 3 of the patients described and was suspected in 2 patients with angiosarcoma of the breast. Angiosarcoma has been observed to develop from irradiated benign angiomata in 3 patients after long intervals. Treatment is fully discussed.

Two cases are added to the recent literature describing a new disease entity in which lymphangiosarcoma originates in the massive arms of women with post-mastectomy lymphoedema.

Sclerosing Lipogranuloma. SMETANA, H. F., and BERNHARD, W. (1950). *Arch. Path.*, **50**, 296. Bibliography.

The authors discuss 14 cases of sclerosing lipogranuloma, taken from the files of the Armed Forces Institute of Pathology, Washington. They assign the term sclerosing lipogranuloma to those cases characterized by tumour-like swelling of subcutaneous fat after trauma of any kind, with basic pathological similarities and not already classified as

recognized clinical entities. Detailed histological appearances are given and comparisons drawn between these lesions and the ones found in allied conditions such as fat necrosis of the breast, Weber–Christian disease, and lipoid pneumonia. The theories of aetiology and pathogenesis of sclerosing lipogranuloma and allied lesions are reviewed. No definite conclusions are reached, but it is suggested that the lipogranulomatous reaction represents a specific process which follows its own course and has distinct clinical and pathological significance.

A. Ackroyd.

Two Cases of Dysgerminoma Ovarii, one Occurring in a Malignant Teratoma in Association with Acute Myelocytic Leukaemia. CHALMERS, J. A. (1950). *J. Obstet. Gynaec. Brit. Emp.*, **57**, 437.

The author describes 2 cases of dysgerminoma ovarii in young girls, one associated with local infiltration and distant metastasis, and the other with myeloid leukaemia. Both cases were fatal, in spite of adequate surgery in the second case. The first case was of pathological interest inasmuch as there was no lymphocytic infiltration of the tumour stroma [Novak (*Gynaecological and Obstetrical Pathology*, 2nd ed.) states that this is a constant feature of the tumour]. The tumour in the second case showed assorted embryonic tissues, of which dysgerminoma was only one of the features.

The author reviews the literature of dysgerminoma ovarii and describes the pathological features of both teratoma and leukaemia. He suggests the possibility of a common origin of teratoma, leukaemia, and dysgerminoma from an undifferentiated mesenchymal cell, a hypothesis which the second case seems to support.

M. Halden Lloyd.

A Histologic Study of Muscles and Nerves in Poliomyelitis. DENST, J., and NEUBUERGER, K. T. (1950). *Amer. J. Path.*, **26**, 863.

In the early stages of poliomyelitis the muscles showed an irregularity in staining associated with a granular fatty degeneration of many fibres. Longitudinal fibrillation and sometimes necrosis were seen. Later many fibres showed atrophic shrinkage (? disease atrophy) and some hypertrophy. Proliferation of sarcolemmal nuclei and formation of muscle giant cells were sometimes conspicuous, but definite evidence of regeneration was absent. In all stages the histological appearances were characteristically pleomorphic. Changes in the nerves (which were obvious by the third day) were less severe than those in muscle, but it seemed that the degree of damage in the muscles and anterior horn cells ran parallel. The muscle changes, however, differed from those of simple central paralysis, and may possibly be due to a direct action of the virus.

D. M. Pryce.

The Histogenesis of Granular-cell Myoblastoma (? Granular-cell Perineural Fibroblastoma). EVERSON PEARSE, A. G. (1950). *J. Path. Bact.*, **62**, 351.

A histological and histochemical examination of 6 specimens of granular-cell "myoblastoma" is described, and the three hypotheses concerning the origin of these lesions—whether from muscle cells, histiocytes, or Schwann cells—are rejected. The granules in the cells are found to stain similarly to granules in fibroblasts. It is suggested that the growths are lipoid-containing granular-cell fibroblastomata.

R. A. Willis.

The Development of Giant-celled Tendon-sheath Tumours and Related Conditions (Chronic Villo-nodular Synovitis and Cutaneous Histiocytoma). SPENCER, H., and WHIMSTER, I. W. (1950). *J. Path. Bact.*, **62**, 411.

Their histological study of specimens taken from 7 cases of villous synovitis and from 34 cases of giant-celled tumour of tendon sheath satisfies the authors [but not the abstracter] that the latter is a non-neoplastic "proliferative response to chronic inflammation." A similar conclusion regarding the pigmented "histiocytoma" or "sclerosing angioma" of the skin is based on a study of 37 of these lesions.

R. A. Willis.

J. clin. Path. (1951), **4**, 253.

AUTO-ANTIBODIES IN ACQUIRED HAEMOLYTIC ANAEMIA

BY

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It is now widely recognized that cases of haemolytic anaemia of spontaneous or endogenous origin may be divided into two broad groups: those determined by a genetical cause resulting in the production of defective red cells, and those not so determined. Among the latter group of acquired haemolytic anaemias are cases in which there is evidence for the presence of antibodies active against the patient's own red cells. That abnormal auto-antibodies might be formed in some cases was suggested by the reports of French authors in the early years of this century. Widal, Abrami, and Brulé (1908a and b; 1909) drew attention to the frequency of autohaemagglutination in cases of *l'ictère hémolytique acquis*, and stressed that this was a distinguishing and characteristic finding. At about the same time Chauffard and Troisier (1908) and Chauffard and Vincent (1909) described as suffering from *ictère hémolysinique* and *hémoglobinurie hémolysinique* patients whose sera were found to contain haemolysins. Although it is true that their reports are somewhat marred by the fact that no account was taken of the existence of the normal isohaemolysins, it seems very probable that the patients' sera did in fact contain abnormal iso-antibodies.

The literature between the pioneer writings of the French haematologists and more recent times is described by Dameshek and Schwartz (1940) in a comprehensive review. They found a number of references to the occurrence of marked autohaemagglutination in acquired haemolytic anaemia, but very few reports of the presence of abnormal haemolysins. They themselves, however, had previously described three patients whose sera appeared to contain abnormal haemolysins (Dameshek and Schwartz, 1938), and a few additional cases have been reported since (Farrar, Burnett, and Steigman, 1940; David and Minot, 1944; Neber and Dameshek, 1947; Dacie, 1949).

The discovery by Coombs, Mourant, and Race (1945) that red cells sensitized with "incomplete" forms of iso-antibodies might be agglutinated by antiglobulin rabbit sera has provided a new tool for the investigation of cases of haemolytic anaemia. Boorman, Dodd, and Loutit (1946) and Loutit and Mollison (1946) reported that the direct Coombs (antiglobulin) test was positive in a number of cases fulfilling the diagnostic criteria of idiopathic acquired haemolytic anaemia, whilst the test was negative in a series of cases of haemolytic anaemia of varying pathogenesis, including several examples of the familial type. This work has since been confirmed.

Thus auto-antibodies have been observed to affect the red cells of patients with acquired haemolytic anaemia in three different ways, i.e., they may cause autohaemagglutination, autohaemolysis, and/or sensitization permitting agglutination by antiglobulin sera. Whether or not these different effects are caused by qualitatively different antibody molecules or are dependent on environmental or other factors is uncertain. Recent work suggests that with Rh antibodies differences in serological behaviour may be correlated with differences in the physico-chemical nature of the antibodies (Hill, Haberman, and Guy, 1949). It is certainly convenient to refer to antibodies as agglutinins or haemolysins, as if these were separate entities, and, while admitting that there may be some doubt about the justification for this, the convention will be followed in this paper.

It is the purpose of this paper to stress the value of trypsinized normal red cells and the red cells of patients with paroxysmal nocturnal haemoglobinuria (P.N.H. red cells) in the detection of abnormal antibodies, to record the antibody patterns that have been observed in various types of haemolytic anaemia, and in particular to present evidence for the quite frequent existence in patients' sera of antibodies capable of causing haemolysis *in vitro*.

The patients have been grouped into three clinico-pathological syndromes: Group I, idiopathic acquired haemolytic anaemia ; Group II, haemolytic anaemia following virus pneumonia ; and Group III, chronic haemolytic anaemia with haemoglobinuria and Raynaud's phenomena. A control group of patients suffering from other types of haemolytic anaemia or blood disorder and a series of normal subjects has also been studied (Group IV).

Twelve patients were considered to belong to Group I. Four patients have died, three despite splenectomy ; several were in partial or almost complete spontaneous or post-splenectomy remission when examined. The four patients of Group II each gave a history of a transient haemolytic anaemia following a pulmonary infection considered to be probably a virus pneumonia. The anaemia of the three patients belonging to Group III was worse in winter time, and each patient gave a history of haemoglobinuria following exposure to severe cold.

A further consideration of the clinical histories of these patients is beyond the scope of this report ; an account of the three patients of Group III is to be reported elsewhere (Ferriman, Dacie, Keele, and Fullerton, 1951). A brief note on the histories of most of the patients studied and certain relevant haematological data are, however, given in Tables I and II.

Three other patients suffering from acquired haemolytic anaemia of unknown origin and type have also been investigated. These cases (Nos. 20–22) have, however, been excluded from Group I because of a complete lack of any evidence for the development of abnormal auto-antibodies ; in particular, Coombs tests were negative and the cold agglutinin titres were normal. They have been placed with some other distinct types of haemolytic anaemia in Group IV.

Materials

Patients' Sera.—Sera were obtained by defibrinating blood at room temperature or by allowing blood to clot at 37° C. and then centrifuging the expressed serum to rid it of suspended red cells whilst still warm. The sera were used fresh or stored frozen at −20° C. for varying periods of time.

Patients' Red Cells.—These were obtained from blood allowed to clot at 37° C., and then washed at least three times in a large volume of warmed saline.

Normal Red Cells.—Cell suspensions were made from the fresh defibrinated blood of a healthy subject of blood group O Rh+ (O Rh− in certain cases). The corpuscles were repeatedly washed in large volumes of saline and used at final concentrations varying from 0.5 to 5%. Group O corpuscles of known Rh, MN.S., *Kell, Lutheran, Lewis*, and *Duffy* types have also been used for detecting the possible presence of immune iso-antibodies.

Complement.—Fresh normal compatible human serum at a final concentration of 1 in 2 was used as a source of complement.

Haemolysis.—This was estimated approximately, after comparing the colour of the supernatant serum with that of a control tube, according to a scale varying from *c* (complete) +++ (almost complete, deep red supernatant) to + (definite, slight haemolysis).

Agglutination.—The presence or absence of agglutination was determined macroscopically with a concave mirror after gently resuspending the deposited corpuscles and comparing the degree of visible agglutination with that of a control tube. The reciprocal of the highest serum dilution giving detectable agglutination was recorded as the titre of the serum.

***p*H.**—Sera were in some instances made more alkaline by the addition of 10% by volume of N/5 or N/10 NaOH or more acid by the addition of 10% by volume of N/10 to N/3 HCl. *p*H was estimated approximately by using thymol blue, phenol red, or bromthymol blue as an indicator, and was checked, when sufficient serum was available, by means of a glass electrode.

Technical Methods

Titration of Cold Agglutinins.—Sera were separated off at 37° C. Serial dilutions of the serum were made in saline using a glass automatic pipette, and equal volumes of a 1% suspension of group O red cells added. A series of 10 tubes giving final concentrations of from 1 in 4 to 1 in 1,024 in addition to a saline control has generally been used. If necessary, the range of serum dilutions has been extended by six or more tubes. Volumes of 0.25 ml. were used as a rule, and the tests carried out in tubes of 10 mm. internal diameter.

The intensity of agglutination was assessed after allowing the suspension to remain undisturbed for at least two hours at 2–5° C. in a refrigerator. In some cases readings were taken at room or higher temperatures before the tubes were chilled. Finally, the racks of tubes were placed in the 37° C. water bath to confirm (or refute) the expected disappearance of agglutination at this temperature

Detection and Titration of Cold Haemolysins.—Acid-sensitive cold haemolysins were demonstrated by the methods used by Dacie (1950c). In its simplest form the test consists of adding an equal volume of fresh normal serum to the patient's serum, acidifying the mixture with a 1/10 volume of N/3.5 HCl and then adding a 1/10 volume of a 30% suspension of normal red cells. The *p*H of the cell-serum suspension should be about 6.5. After allowing the tubes to stand for one hour at about 15° C., the suspensions are placed in a water bath at 30° C., the temperature of which is then gradually raised to 37° C. The tubes are centrifuged and inspected for haemolysis after one hour at 37° C.

Paroxysmal Nocturnal Haemoglobinuria Red Cells and Trypsinized Normal Red Cells.—There also were serial dilutions of the patient's serum made in fresh normal compatible serum and an equal volume of a 2% saline suspension of red cells added.

After the tubes have been allowed to stand for one hour at about 15° C., they were warmed for a further hour at 30–37° C.

Detection and Titration of Warm Haemolysins.—Tests using normal corpuscles, normal "trypsinized" corpuscles, and P.N.H. red cells were employed.

Tests with Normal Corpuscles.—The patient's serum was first acidified to a *p*H of approximately 6.5 by the addition of a 1/10 volume of N/3.5 HCl. It was then placed in a water bath at 37° C. and a 1/10 volume of a warmed 30% saline suspension of normal red cells added. The suspension was centrifuged after one hour. If no haemolysis had resulted, the supernatant serum was removed and the corpuscles resuspended in a volume of fresh undiluted normal serum acidified to *p*H 6.8–7.0 by the addition to it of a 1/10 volume of N/5 HCl. The tube was again inspected for haemolysis after a further hour at 37° C.

Tests with Normal Corpuscles Modified by Trypsin (Morton and Pickles, 1947).—Normal corpuscles were "trypsinized" by the addition of 1 volume of washed packed red cells to 5 volumes of diluted trypsin solution (1 volume of 1.0% crystalline trypsin (Armour) in N/20 HCl added to 9 volumes of isotonic phosphate buffer at *p*H 7.7). The suspension of corpuscles in the enzyme solution was warmed at 37° C. for 60 minutes, then centrifuged, and the red cells washed in two changes of saline. The corpuscles were generally used at a final concentration of 1%.

Tests for the haemolysis of trypsinized normal red cells were carried out by adding equal volumes of a 2% suspension of enzyme-treated red cells to two- or four-fold dilutions of the patient's serum in fresh normal unacidified compatible serum. The tubes were centrifuged after one hour at 37° C. and then inspected for haemolysis. If the serum is acidified, as for tests with normal unmodified corpuscles, haemolysis is slightly increased. Acidified serum was not, however, employed as a routine..

Tests with P.N.H. Red Cells.—Three patients were available; two were of blood group ORh+ and one was of group ARh+. The red cells from these patients were used unmodified or trypsinized as described above, except that the time of the exposure to the enzyme was reduced to 15 minutes. Undiluted unacidified fresh normal serum has been used as the diluent for the patients' sera.

Paroxysmal nocturnal haemoglobinuria red cells, even if exposed to trypsin for as little as 15 minutes, undergo a small but variable amount of haemolysis in some normal unacidified human sera. It is necessary, therefore, to select a serum for use as diluent which produces no haemolysis or at the most a trace of haemolysis on its own account.

Detection and Titration of Incomplete Warm Antibodies.—Several methods were used, including the direct and indirect Coombs tests.

Direct Coombs Test.—The method employed by Mollison and Cutbush (1949) was used. The patient's corpuscles were collected directly into isotonic citrate-saline or obtained from blood allowed to clot at 37° C. They were washed in at least three changes of a large volume of saline warmed to 37° C. A 10% suspension in saline was then mixed upon a white translucent tile with an equal volume of well-absorbed antiglobulin rabbit serum diluted to its point of maximum avidity. Antiglobulin sera were prepared according to the methods of Proom (1943) and Wootton (1950).

Indirect Coombs Test.—A 5% suspension of normal group O corpuscles in the patient's serum was made by adding to it a 1/10 part of a 50% suspension of washed red cells. The suspension was warmed to 37° C. for two hours and the corpuscles then washed three times in a large volume of warmed saline. Tests for agglutination by antiglobulin serum were carried out as detailed above. Acidified serum was also used, as suggested by Gardner (1949); a 1/10 volume of N/3.5 HCl was added to a volume of serum before adding the suspension of corpuscles. Tests for the presence of immune

iso-antibodies were carried out on unacidified sera using corpuscles of known Rh, MN.S., *Kell, Lutheran, Lewis,* and *Duffy* types.

Preparation of Antibody-containing Eluates of Red Cells.—Kidd's (1949) method was followed, except that the time of elution at *p*H 3.2–3.4 was extended from 10 to 15 minutes to 30 to 45 minutes.

The eluates were tested for antibody content in three ways: (1) by making serial dilutions in saline and adding equal volumes of a 2% suspension of washed normal red cells, warming to 37° C. for two hours, and then carrying out tests for adsorbed incomplete antibody using an antiglobulin serum ; (2) by making serial dilutions in saline and adding equal volumes of a 2% suspension of trypsinized normal red cells, warming to 37° C. for two hours, and then inspecting for agglutination ; and (3) by making a series of dilutions of the eluate in fresh normal compatible serum and adding equal volumes of 2% suspensions of trypsinized normal red cells, P.N.H. red cells, and trypsinized P.N.H. red cells respectively and inspecting for haemolysis after one hour at 37° C.

The tests for haemolysis were also carried out in the following way. The cell-eluate suspensions were lightly centrifuged after warming for one hour at 37° C. and the supernatants then replaced by volumes of fresh normal serum. The tubes were finally inspected for haemolysis after a further hour at 37° C.

Agglutination Tests with Serum-bovine Albumin Diluent.—The method was slightly modified from that described by Neber and Dameshek (1947). Serial dilutions of the patient's serum were made in fresh compatible normal serum and a 2% suspension of normal corpuscles in 30% bovine albumin (Armour) added to each tube. The tubes were kept at 37° C. for two hours and agglutination then read off macroscopically after gently resuspending the deposited corpuscles.

Agglutination Tests with Trypsinized Normal Corpuscles.—Tests for the agglutination of trypsinized normal red cells were carried out by adding equal volumes of a 2% suspension of enzyme-treated red cells to two- or four-fold dilutions of the patient's serum in saline. The presence or absence of agglutination was ascertained after two hours incubation at 37° C.

Titration of Complement.—Serial dilutions of freshly obtained sera were made in saline so as to give a range of serum dilutions of 1 in 2 to 1 in 64. Equal volumes of a 2½% suspension of sensitized sheep cells were then added to each tube. The tubes were centrifuged after 30 minutes at 37° C., and the amount of the haemolysis determined photoelectrically. The titre of complement was recorded as the reciprocal of the final concentration of serum causing 50% haemolysis (Mayer, Eaton, and Heidelberger, 1946).

The sheep cells used were sensitized with a rabbit anti-sheep cell serum diluted to 1 in 1,000 in saline (approximately 1½ M.H.D.).

Other Haematological Estimations.—Haemoglobin estimations, reticulocyte counts, and osmotic fragility determinations were carried out by methods described by Dacie (1950a).

Results

The results of applying the methods described above for the detection and titration of auto-antibodies are summarized in Tables I, II, and III.

Cases of Acquired Haemolytic Anaemia (Groups I, II, and III).—The results for these groups are discussed in detail.

Cold Agglutinins.—In seven out of the 12 patients of Group I the titre of cold agglutinins was abnormal, ranging from 256 to 1024 ; in one other case it was at the upper limit of the normal range (64). The agglutinin titres and thermal ranges

TABLE I

RESULTS OF SEROLOGICAL AND OTHER INVESTIGATIONS IN IDIOPATHIC ACQUIRED HAEMOLYTIC ANAEMIA (GROUP I)

Case No., Sex, Age, Date	Non-specific Cold Agglutinins	Non-specific Warm Haemolysins				Non-specific Incomplete Warm Antibodies		
							Indirect Coombs Test	
	Normal Red Cells in Saline (titre 2–5° C.)	Normal Red Cells in Acidified Serum (titre 37° C.)	Trypsinized Normal Red Cells in Serum (titre 37° C.)	P.N.H. Red Cells in Serum (titre 37° C.)	Trypsinized P.N.H. Red Cells in Serum (titre 37° C.)	Direct Coombs Test (37° C.)	Unacidified Serum (37° C.)	Acidified Serum (37° C.)
1. F., 18 March, 1947	512	8	256	64	1,024	++	+	+
2. M., 35 6.12.48	512	0	0	0	0	+	0	+
3. F., 27 .. 18.4.49	512	2 (trace haemolysis)	32	0	64	+	0	+
4. F., 22 .. 18.10.49	256	0	64	32	512	+	+	++
5. F., 56 .. (*a*) 23.10.49	1,024	2 (trace haemolysis)	32	32	512	+	0	+
(*b*) 27.10.50	256	0	4	16	256	+	0	0
6. F., 50 .. 30.10.50	4	0	0	0	0	+++	±	+
7. F., 72 .. 12.10.50	64	0	0	0	0	+	0	+
8. M., 55 .. 31.10.50	4	0	0	0	0	+++	0	0
9. M., 42 .. 1.11.50	2	0	0	0	0	+	0	0
10. M., 39 .. 21.10.50	4	0	0	0	0	+	0	0
11. F., 54 .. (*a*) 19.10.50	1,024	2 (trace haemolysis)	128	512	8,000	++	0	+
(*b*) 1.2.51	1,024	0	256	1,024	8,000	+	0	+
12. F., 73 .. (*a*) 26.10.50	512	2 (trace haemolysis)	16	4	256	++	0	+
(*b*) 31.1.51	1,024	2 (trace haemolysis)	32	32	512	+	0	+

TABLE I (*continued*)

Non-specific Incomplete Warm Antibodies				Specific Immune Iso-antibodies	Serum Complement	Other Data
Agglutination of Normal Red Cells in Serum-albumin (titre 37° C.)	Agglutination of Trypsinized Normal Red Cells in Saline (titre 37° C.)	Red Cell Eluates (Kidd)				
		Indirect Coombs Test (titre 37° C.)	Agglutination of Trypsinized Normal Red Cells in Saline (titre 37° C.)	Indirect Coombs Test (Unacidified Serum)	Units	
16	4	—	—	Trace (unidentified)	70	Splenectomy 5 years previously. Severe relapse. Hb 5·0 g. Reticulocytes 75%. Died
16	64	—	—	0	100	Very severely ill; terminal haemoglobinuria. Hb 6·6 g. Reticulocytes 28%. Died
8	4	—	—	0	—	Severely ill; died despite splenectomy
8	16	—	—	0	—	Severely ill; Hb 2·5 g. Reticulocytes 38%. Died despite splenectomy
0	2	—	—	—	—	Severely ill; haemoglobinuria. Hb 4·1 g. Reticulocytes 0·5%
0	0	0	4	0	100+	1 year after splenectomy. Hb 14·8 g. Reticulocytes 0·6%
16	16	128	128	0	100+	In remission. Splenectomy 3 years previously. Hb 14·5 g. Reticulocytes 2·2%
0	0	—	—	0	—	In spontaneous remission. Hb 14·5 g. Reticulocytes 1%
0	4	64	512	0	100+	In remission. Splenectomy 6 months previously. Hb. 14·7 g. Reticulocytes 7%
2	1024	8	32	++ (anti-c)	100+	In remission. Splenectomy 9 months previously. Hb 15·4 g. Reticulocytes 1%
0	0	—	—	0	—	In remission. Splenectomy 3 years previously. Hb 16·3 g. Reticulocytes 1%
16	8	8	16	0	10–32	Moderately active haemolysis. Hb 9·5 g. Reticulocytes 6%
8	4	—	—	0	27	9 weeks after splenectomy. Hb 14·5 g. Reticulocytes 3·5%
0	0	16	16	0	10	Moderately active haemolysis. Hb 5·0 g. Reticulocytes 11%
4	0	—	—	+ (? anti-E)	30	10 weeks after splenectomy. Hb 13·2 g. Reticulocytes 1%

— signifies no observation. 0 signifies a negative result.

RESULTS OF SEROLOGICAL AND OTHER INVESTIGATIONS IN HAEMOLYTIC ANAEMIA FOLLOWING VIRUS PNEUMONIA, CHRONIC HAEMOLYTIC ANAEMIA WITH HAEMOGLOBINURIA AND RAYNAUD'S PHENOMENA, AND A CONTROL GROUP OF OTHER TYPES OF HAEMOLYTIC ANAEMIA OR BLOOD DISORDER AND NORMAL SUBJECTS

Case No., Sex, Age, Date	Non-specific Cold Agglutinins	Non-specific Warm Haemolysins				Non-specific Incomplete Warm Antibodies		
							Indirect Coombs Test	
	Normal Red Cells in Saline (titre 2–5° C.)	Normal Red Cells in Acidified Serum (titre 37° C.)	Trypsinized Normal Red Cells in Serum (titre 37° C.)	P.N.H. Red Cells in Serum (titre 37° C.)	Trypsinized P.N.H. Red Cells in Serum (titre 37° C.)	Direct Coombs Test (37° C.)	Unacidified Serum (37° C.)	Acidified Serum (37° C.)
GROUP II								
13. F., 22 .. 8.9.50 ..	16,000	0	0	16	512	+	0	0
14. F., 73 .. 14.11.50	4,000	0	0	16	1,024	+	0	+
15. F., 33 .. 28.11.50	4,000	0	64	0	256	+	0	0
16. M., 35 .. 12.12.50	2,000	0	0	16	64	+	0	0
GROUP III								
17. F., 67 .. 10.5.50	64,000	0	16	0	16	+	0	0
18. M., 79 .. 16.12.49	128,000	0	0	0	64	+	0	0
19. F., 64 .. 23.5.50	64,000	0	0	64	512	+	0	+
GROUP IV								
20–22 ..	8	—	0	0	0	0	—	—
23–29 ..	16	—	0	0	0	0	—	—
30. F., 27 .. 20.11.50	8	—	0	16	16	0	—	—
31. F., 46 .. 2.10.50	8	0	0	0	0	0	0	—
32–33 ..	4–8	0	0	0	0	0	0	—
34. F., 52 .. 12.10.50	4	—	0	0	0	+	—	—
35–36 ..	4–8	—	0	0	0	+	—	—
37–54 ..	—	—	0	0	—	—	—	—
55. F., 72 .. 20.10.50	4	—	16	0	64	0	—	—
56–161 ..	—	—	0	0	—	—	—	—

TABLE II (*continued*)

Non-specific Incomplete Warm Antibodies				Specific Immune Iso-antibodies	Serum Complement	Other Data
Agglutination of Normal Red Cells in Serum-Albumin (titre 37° C.)	Agglutination of Trypsinized Normal Red Cells in Saline (titre 37° C.)	Red Cell Eluates (Kidd)		Indirect Coombs Test (Unacidified Serum)	Units	
		Indirect Coombs Test (titre 37° C.)	Agglutination of Trypsinized Normal Red Cells in Saline (titre 37° C.)			
GROUP II						
—	0	—	—	—	—	Presented as puerperal anaemia
—	0	—	—	—	110	In a severe haemolytic episode with jaundice
—	0	—	—	—	160	Recovering from an acute episode of haemolysis. Hb 10·5 g. Reticulocytes 18%
—	0	—	—	—	70	In an acute episode of haemolysis
GROUP III						
—	0	—	—	—	90	In remission (warm weather). Hb 14·8 g. Reticulocytes 1%
—	0	—	—	—	—	Cyanosis of extremities, pallor, and jaundice. Hb 8·9 g. Reticulocytes 20%
—	0	—	—	—	10	In partial remission (warm weather). Hb 8·3 g. Reticulocytes 14%
GROUP IV						
—	0	—	—	—	—	Adults affected with haemolytic anaemia of unknown origin and type. Osmotic fragility normal
—	0	—	—	—	—	Congenital haemolytic anaemias (hereditary spherocytosis, etc.)
—	0	—	—	0	110	Atypical congenital haemolytic anaemia (relapse after splenectomy). Hb 7·5 g. Reticulocytes 50%
—	0	0	0	+ (anti-Kell)	70	Paroxysmal nocturnal haemoglobinuria. Transfused many times
—	0	—	—	0	—	Paroxysmal nocturnal haemoglobinuria. Transfused many times
—	0	—	—	—	<4	Haemolytic anaemia with lymphadenoma. Hb 7 g. Reticulocytes 4%
—	0	—	—	—	—	Disseminated lupus erythematosus. Slight to moderate anaemia
—	0	—	—	—	—	Miscellaneous anaemias and leukaemias
—	0	—	—	—	100+	Pernicious anaemia. In remission
—	0	—	—	—	—	Non-anaemic hospital patients and normal subjects

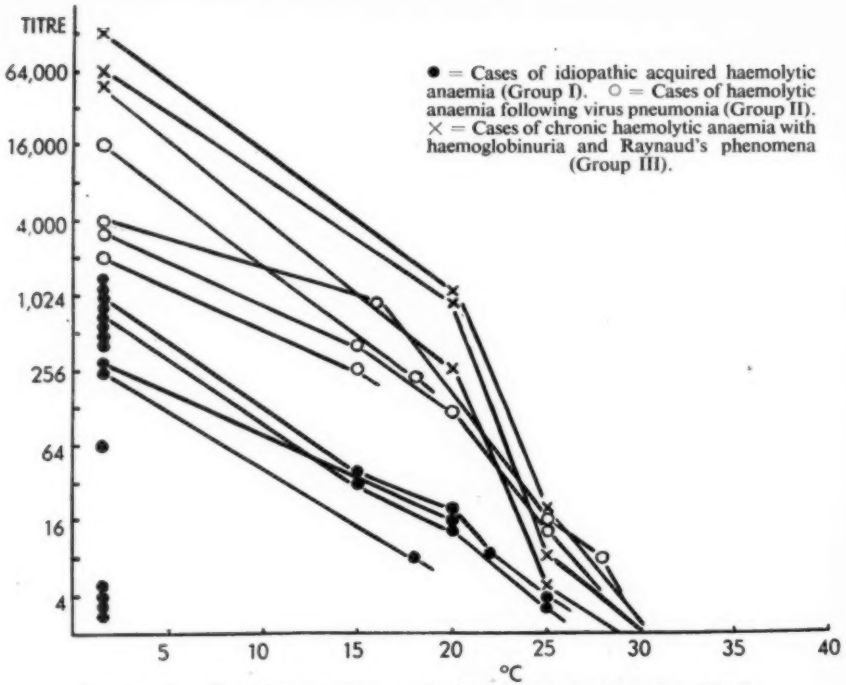

FIG. 1.—The effect of temperature on the titre of non-specific cold agglutinins.

were higher in the patients with haemolytic anaemia following virus pneumonia (Group II) and higher still in those patients (Group III) suffering from chronic haemolytic anaemia with haemoglobinuria and Raynaud's phenomena (Fig. 1).

Incomplete forms of cold antibodies were demonstrated in all the sera that were tested (Dacie, 1950b). In some cases the titres were greater than are found in health.

Cold Haemolysins.—Using the acidified serum technique, haemolysis of normal red cells in the presence of fresh serum was readily observed with the sera of all the patients of Groups II and III, and a cold antibody in weak concentration was similarly detected in the serum of one patient of Group I (Case 5). Titration of cold haemolysins using P.N.H. red cells gives very high titres, as already recorded (Dacie, 1950c). The sera which gave the highest titres of the present series belonged to patients from Group III. Details of the results of titration at 37° C. and at 15° C. followed by a period at 37° C. are given in Table III.

The titres observed when trypsinized normal red cells were used in the place of P.N.H. red cells were less high. In Case 15 it is interesting to note that a temperature effect was observed when P.N.H. red cells were used but not when trypsinized normal red cells were substituted (Table III); thus, in this case there appears to be

evidence for the existence of a fraction of antibody indifferent to temperature in addition to the cold antibody.

The effect of temperature was tried with most of the sera of the patients of Group I; preliminary cooling to 15° C., or below, before warming to 37° C. did not increase the amount of haemolysis that was observed. The results obtained with the sera of Cases 11 and 12 are included in Table III.

Warm Haemolysins.—Using normal corpuscles, haemolysis at 37° C. was easily demonstrated only in the first patient of this series, fuller details of whom have already been reported (Dacie, 1949). Using the same technique, small amounts of haemolysis were produced by the sera of Cases 3, 5, 11, and 12, but only when the sera were acidified.

By using trypsinized normal red cells, definite indications of the presence of a haemolytic antibody active at 37° C. and requiring the presence of fresh serum complement were obtained with the sera of six out of the 12 patients of Group I. These patients (three after splenectomy) were suffering from negligible to extremely serious haemolysis *in vivo* at the time the serum samples were obtained. The serum from the other seriously ill patient of this series (Case 2) gave a negative result. Of the patients belonging to Groups II and III, the serum of one (Case 15) haemolysed trypsinized normal red cells at 37° C.

Paroxysmal nocturnal haemoglobinuria red cells similarly underwent haemolysis at 37° C. in the sera of five of the 12 patients of Group I, in the sera of three of the four patients of Group II, and in one of the three sera of Group III. When trypsinized, P.N.H. red cells become extremely sensitive reagents. They were haemolysed at 37° C. by six of the 12 sera of Group I, in most instances to high titres, and by all the sera of Groups II and III.

It is interesting to note the lack of parallelism between the behaviour of trypsinized normal red cells and P.N.H. red cells with different sera; e.g., the increased sensitivity of P.N.H. red cells compared with trypsinized normal red cells in Case 5b and the opposite relationship in Case 12a, both titrations being carried out side by side at the same time.

Direct Coombs Tests.—In all of the 19 cases of acquired haemolytic anaemia of Groups I, II, and III, antiglobulin tests carried out on the patients' corpuscles were found to be positive. The apparent strength of the test varied from very strong reactions (agglutination within a few seconds) to quite weak reactions as in Case 5a. The strength of the reactions recorded in Table I have been transcribed from the records made at the time the patients were examined, a period extending well over two years. Of necessity various samples of antiglobulin sera were used, and the recorded strength of the reactions can be taken as only roughly quantitative.

The tests were weakly positive in the case of three patients of Group IV (Cases 34–36); in these patients all other tests for the presence of abnormal anti-red cell antibodies were, however, negative. In three patients with acquired haemolytic anaemia of idiopathic type, Cases 20–22, the reactions were negative; in two of the patients the tests were carried out repeatedly, with varying dilutions of antiglobulin sera, so as to overcome possible prozone inhibition phenomena (van Loghem, 1950; van Loghem, Kresner, Coombs, and Fulton Roberts, 1950).

The significance of the positive tests obtained with the corpuscles of the patients of Groups II and III is possibly different from that pertaining to Group I. In

Group I it seems certain that a warm antibody (i.e., an antibody whose activity is not significantly increased by reduction of temperature below 37° C.) is involved. In Groups II and III the direct antiglobulin reaction probably results from the presence of incomplete cold antibody irreversibly adsorbed due to minor degrees of cooling *in vivo*. The corpuscles were in all cases kept at 37° C. before being exposed to the action of the antiglobulin serum so as to avoid the possibility of the adsorption *in vitro* of incomplete cold antibody.

Indirect Coombs Tests.—Positive indirect antiglobulin tests apparently due to sensitization by non-specific antibodies were obtained with corpuscles exposed to the sera of all the patients of Group I in whom haemolysis was proceeding actively *in vivo* (Cases 1, 2, 3, 4, 5, 11, and 12); in five of these patients a positive result was, however, obtained only when their sera were acidified. In two patients in clinical remission (Cases 6 and 7) the reactions were also positive. In three instances definite reactions in acidified sera were associated with weaker sensitizations in unacidified serum; in one of these patients traces of unidentified specific iso-antibodies were probably present in her serum in addition to non-specific antibodies (Case 1).

The indirect antiglobulin test was positive with the acidified serum of one out of the four patients of Group II and with that of one out of the three patients of Group III.

Activity of Red Cell Eluates.—Eluates were made by Kidd's method from the corpuscles of six patients of Group I. Four of the patients were in remission. In each case antibodies could be detected by the indirect antiglobulin test, and the eluates were also capable of agglutinating trypsinized normal red cells. The eluates had, however, no haemolytic activity, even for trypsinized P.N.H. red cells.

Agglutination of Normal Red Cells in Serum-albumin at 37° C.—In none of the patients of this series was a non-specific agglutinin demonstrated to be active at 37° C. in saline dilutions of patients' sera. In eight patients of Group I there was, however, definite evidence for the presence of non-specific incomplete antibodies detectable at 37° C. by titration with normal corpuscles in a serum-albumin medium.

In several patients quite strong auto-agglutination of whole blood was observed to take place at 37° C. This phenomenon is probably due to agglutination of strongly sensitized corpuscles in whole plasma or serum. This was particularly striking with the blood of Case 8. Normal corpuscles were not, however, agglutinated by this patient's serum either at 37° C. or at room temperature. In other instances where cold panagglutinins were present at high concentrations (titres>256), auto-agglutination was obvious at room temperature, and in some cases it did not disappear completely until the temperature was raised to 30–32° C. (Fig. 1).

Agglutination of Trypsinized Normal Red Cells at 37° C.—The sera of nine out of the 12 patients of Group I caused the agglutination of trypsinized normal red cells at 37° C. This technique was found to be in general about as sensitive as titration in serum-albumin using normal red cells; with trypsinized normal red cells the end points are, however, much more easily read (at 37° C.). The serum from Case 9 was unusual; in addition to anti-c it appeared to contain a non-specific antibody which agglutinated trypsinized normal red cells to a far higher titre than it agglutinated normal red cells in serum-albumin.

Agglutination of trypsinized red cells at 37° C. was not caused by any of the sera of the patients of Groups II, III, and IV.

Titration of Complement.—In three patients (Cases 11, 12, and 19) the content of complement in the sera was found to be significantly lowered; it was markedly reduced in the serum of Case 34 (haemolytic anaemia with lymphadenoma).

Control Cases (Group IV)

Tests for haemolysins using trypsinized normal red cells and unmodified P.N.H. red cells were carried out with the sera from a mixed series of cases of haemolytic anaemia not considered to belong to Groups I, II, and III, as well as with the sera of a group of patients suffering from various anaemias and leukaemias and with sera from normal healthy subjects and from hospital patients not considered to be suffering from blood diseases. The tests were negative with two exceptions, Cases 30 and 55 (Table II).

Case 30 was a patient with an atypical non-spherocytic congenital haemolytic anaemia which persisted after splenectomy. Her serum contained a thermostable haemolytic factor which caused the haemolysis of P.N.H. red cells at 37° C., even

TABLE III

EFFECT OF TEMPERATURE ON THE HAEMOLYSIS OF VARIOUS TYPES OF RED CELLS BY THE SERA OF PATIENTS SUFFERING FROM ACQUIRED HAEMOLYTIC ANAEMIA

Case No.		Normal Red Cells in Acidified Serum (*p*H 6.5)	Trypsinized Normal Red Cells (titre)	P.N.H. Red Cells (titre)	Trypsinized P.N.H. Red Cells (titre)
11	37° C.	Trace	128	256	8,000
	15° C., then 37° C.	,,	128	256	—
12	37° C.	,,	64	16	512
	15° C., then 37° C.	,,	64	16	—
13	37° C.	0	0	16	512
	15° C., then 37° C.	+	32	1,024	—
14	37° C.	0	0	16	1,024
	15° C., then 37° C.	+	16	1,024	—
15	37° C.	0	64	0	256
	15° C., then 37° C.	+	64	1,024	—
16	37° C.	0	0	16	64
	15° C., then 37° C.	+	0	512	—
17	37° C.	0	16	0	16
	15° C., then 37° C.	++	256	16,000	—
18	37° C.	0	0	0	64
	15° C., then 37° C.	++	1,024	32,000+	—
19	37° C.	0	0	64	512
	15° C., then 37° C.	++	1,024	32,000	—

Cases 11 and 12, idiopathic acquired haemolytic anaemia; Cases 13–16, haemolytic anaemia following virus pneumonia; Cases 17–19, chronic haemolytic anaemia with haemoglobinuria and Raynaud's phenomena.

when the serum was unacidified. Trypsinized normal red cells were not affected, nor were trypsinized P.N.H. red cells haemolysed to a higher titre than were the unmodified P.N.H. red cells. There were no other evidences of abnormal auto-antibody formation ; the Coombs test was negative, the cold agglutinin titre normal, and the survival of transfused normal red cells unimpaired. Case 55 had suffered from pernicious anaemia but was in complete remission following vitamin B_{12} therapy when investigated. Her serum contained a thermolabile factor which haemolysed at 37° C. trypsinized normal red cells but not unmodified P.N.H. red cells. The significance of these observations is obscure ; the haemolytic factors present in the sera of these two cases differed in their behaviour from each other and from the haemolytic antibodies demonstrated in the sera of the patients with acquired haemolytic anaemia.

Discussion

The methods outlined in this paper have provided useful information concerning the pathogenesis of some types of haemolytic anaemia. In particular, the existence of abnormal non-specific haemolytic antibodies in the sera of some of the patients can scarcely be doubted. Trypsinized normal red cells and P.N.H. cells are clearly valuable laboratory reagents, the former the more so because they are readily available.

The use of unusually sensitive red cells in the detection of antibodies in cases of haemolytic anaemia is relatively new. Trypsinized red cells have been observed to be agglutinated by the sera of patients with haemolytic anaemia (Wright, Dodd, and Bouroncle, 1949 ; Wheeler, Luhby, and Scholl, 1950) and have been widely used in the detection of incomplete iso-antibodies, particularly anti-Rh. That haemolysis may be observed with sera from cases of haemolytic anaemia does not seem, however, to have been reported. Similarly, although the very great sensitivity of P.N.H. red cells to haemolysis by cold haemolysins has already been reported (Dacie, 1950c), the fact that they may undergo haemolysis at 37° C. in the diluted unacidified serum of certain patients with acquired haemolytic anaemia does not seem to have been recognized. There appear to be two possible reasons for the haemolysis that has been observed ; either that trypsinized normal red cells and P.N.H. red cells are sufficiently sensitive to demonstrate antibodies existing in concentrations too low to affect normal corpuscles or, alternatively, that they are acted upon by higher-titre antibodies to which normal corpuscles are almost completely insensitive. It can be only a matter of speculation at the moment which of these possibilities is correct. The remarkably high titres of warm antibodies in some of the patients' sera, Case 11, for example, certainly suggest that the patients' red cells (and normal unmodified red cells) may be remarkably insensitive to these antibodies. Acidification of serum, as used by Dacie (1949, 1950c) and Gardner and Harris (1950), seems capable of increasing the sensitivity of normal red cells to both warm and cold non-specific antibodies. However, the apparent haemolytic titres are far less than those which may be observed using trypsinized normal red cells or P.N.H. red cells. Acidification of sera slightly increases the amounts of haemolysis which result when trypsinized red cells are used, but this seems an unnecessary refinement for diagnostic purposes, and will lead to complications if P.N.H. red cells are used. The acidification of serum when carrying out indirect Coombs tests (Gardner, 1949) is, however, clearly an important practical advance.

The Pattern of Antibodies in Haemolytic Anaemia Sera.—An interesting feature of the behaviour of the sensitive cells used in this study has been the different patterns of results which have been obtained with different sera, suggesting perhaps individual variations in the molecules of antibody globulin. The reciprocal relationship, already referred to, between the sensitivity of trypsinized normal red cells and P.N.H. red cells to haemolysis at 37° C. by two sera (Nos. 5b and 12a), tested side by side, illustrates this. Case 2 illustrates a further variation. This patient died of his disease and could never be made fit enough for splenectomy to be attempted; although his serum strongly agglutinated trypsinized normal red cells, haemolysis *in vitro* in the presence of complement did not take place. Case 11 is in complete contrast; her serum was powerfully haemolytic at 37° C., but only weakly agglutinated trypsinized normal red cells.

The individuality of serum antibodies is also demonstrated by the results obtained with the sera of Group II. The antibodies present in the sera of the patients of this group were essentially cold ones, but with some of the sera, but not with all, haemolysis took place at 37° C. This appears to be due generally to cold antibodies being present at such high concentrations that their thermal range extends to 37° C. when tested for by sufficiently sensitive red cells. In Case 15, however, the haemolysis of trypsinized normal red cells was not increased by preliminary cooling; presumably, therefore, there was in this serum a warm component of antibody, detectable by means of trypsinized normal red cells, in addition to cold haemolysin detectable by P.N.H. red cells (Table III). In contrast, the antibody detectable by trypsinized normal red cells in the serum of Case 14 appeared to be a cold one. In Case 16 the cold antibody was easily demonstrated with P.N.H. red cells, but not at all with trypsinized normal red cells. Similar subtle differences can be noted if the results obtained with the sera from the patients of Group III are studied (Table III).

Significance of Different Types of Non-specific Antibody.—The role of haemolysins as an important cause of haemolysis *in vivo* in cases of acute haemolytic anaemia has been vigorously propounded by Dameshek and his collaborators (Dameshek and Schwartz, 1938, 1940; Dameshek and Miller, 1943). A difficulty in accepting this hypothesis has been the rarity with which the presence of an abnormal autohaemolysin has been unequivocally demonstrated in the laboratory. Ham and Castle (1940) and Wagley, Shen, Gardner, and Castle (1948) have emphasized that auto-agglutination occurring *in vivo* might itself be a cause of haemolysis, and the spleen might be particularly important in this respect owing to the slow circulation through it and the opportunities for auto-agglutination and trapping of agglutinates which might result from this.

The discovery by the application of the Coombs test that incomplete forms of auto-antibodies were frequently present in cases of acquired haemolytic anaemia suggested that this type of antibody might be the elusive haemolytic factor. It was soon realized, however, that, although the direct Coombs test was usually strongly positive during periods of intense haemolysis, this was not necessarily true, and that patients might be observed in complete or almost complete clinical remission, in whom the test was markedly positive (Loutit and Mollison, 1946; Evans and Duane, 1949; our Cases 6 and 8). It was also not easy to understand how cells sensitized by an antibody only capable of causing agglutination under artificial conditions *in vitro* could be the cause of haemolysis *in vivo*. Wagley *et al.* (1948) observed

that the red cells obtained from the spleens removed from three patients suffering from acquired haemolytic anaemia were more strongly agglutinated by an antiglobulin serum than those obtained from samples of peripheral blood. They considered that this higher concentration of antibody might result in local auto-agglutination with stagnation of blood within the spleen and subsequent haemolysis.

The potent antibody-containing eluates which have been obtained from the red cells of several patients of the present series emphasize that incomplete antibodies are by themselves of minor importance in causing haemolysis *in vivo*, at least in patients whose spleens have been removed. The most potent eluates were obtained from two patients in almost complete remission (Cases 6 and 8), whose corpuscles gave strongly positive direct Coombs tests. It is noteworthy perhaps that antibodies capable of causing haemolysis could not be demonstrated in the eluates of these cases or in eluates from the corpuscles of other patients in whom haemolysis was proceeding rapidly, and in whose sera haemolytic antibodies could be demonstrated. The reason for this awaits an explanation. Possibly the haemolytic antibodies are hardly adsorbed at all by normal corpuscles under physiological conditions, or, if they are, the adsorption of a very few molecules is sufficient to cause haemolysis to take place rapidly ; both explanations, if correct, would reduce the chance of obtaining in an eluate of washed red cells antibodies of haemolytic potency. The fact that these eluated antibodies failed to cause haemolysis under what appeared to be optimal conditions, although capable of producing agglutination, seems in favour of the conception that the incomplete and haemolytic antibodies are separate entities.

The observations recorded in this paper thus appear to confirm the relative unimportance of incomplete antibodies. By demonstrating the relative frequency of haemolytic antibodies, they reopen the question of the importance of haemolysins. The findings certainly indicate the existence of haemolytic antibodies in the sera of certain patients affected with acquired haemolytic anaemia if sensitive methods are used to detect the antibodies. It must be conceded that these observations do not necessarily prove that the antibodies play an important, or even any, part in causing haemolysis *in vivo* ; for one thing there seems to be no exact correlation between antibody content and the clinical evidence of haemolysis. On the other hand, it is difficult to believe that the ability of the antibodies to cause haemolysis *in vitro* is entirely without significance. However, it is certainly not claimed, nor is it thought probable, that the haemolytic antibodies are the sole cause of haemolysis in the types of case under review. The possibility that auto-agglutination *in vivo* may be of some importance or that the presence of adsorbed incomplete antibody may predispose a red cell to phagocytosis or shorten its life in some other way cannot be denied, and it is quite clear, judging from the frequent (but not invariable) beneficial results of splenectomy (Welch and Dameshek, 1950 ; our Cases 5, 11, and 12) that the spleen may be of considerable importance also. Whether this is solely due to the organ exerting a haemolytic function depending upon circulatory stagnation or the phagocytic action of its reticulo-endothelial cells acting on corpuscles damaged by antibodies or any other mechanism, is not quite clear.

Effect of Splenectomy on Antibody Titres.—There seem to be few studies available of antibody concentrations following splenectomy in man for acquired haemo-

lytic anaemia. Evans and Duane (1949) for instance, studied two patients before and after splenectomy; in each case a diminution in the rate of haemolysis was associated with a striking reduction in the agglutinability of the patients' red cells by antiglobulin sera. Three of the patients in this series were studied in some detail before and after splenectomy. Haemolytic antibodies could still be demonstrated in the serum of Case 5 a year after splenectomy although she appeared to be in almost complete remission. There was a fall in titre to between one-quarter and one-half of its original value. The cold agglutinin titre also had fallen to one-quarter of its former value. In Cases 11 and 12 little or no change in the antibody titres could be demonstrated in the first 10 or 11 weeks after splenectomy.* Both patients were, however, greatly improved clinically; a red cell survival study showed that transfused blood was surviving in Case 11 at least twice as long as it was before splenectomy. In these patients, therefore, it appears that the spleen was an important haemolytic organ, but a minor source of antibody. That the spleen is, however, capable of being an important source of antibody both in animals and man is shown by the recent studies of Rowley (1950a and b) and Wolfe, Norton, Springer, Goodman, and Herrick (1950). Further studies are necessary before the relative importance of these two aspects of splenic activity can be reliably assessed; probably both play a part in cases of human haemolytic anaemia, the relative importance of each varying from case to case.

Importance of Osmotic Fragility.—Another factor that must play some part in the production of haemolysis *in vivo* is abnormal spherocytosis. The spleen is to some extent involved in this phenomenon too. An increase in osmotic fragility is generally, but not invariably, found in acquired haemolytic anaemia. Moderate to marked increases were found in all the patients of Group I of this series in whom the estimation was carried out and in whom haemolysis *in vivo* was markedly increased (Cases 1, 2, 4, and 5). On the other hand the fragility of the red cells of Cases 11 and 12 in whom haemolysis *in vivo* was moderately severe was only slightly increased, and when Case 6 was first seen (in 1947 before splenectomy), fragility was within the normal range, although haemolysis was proceeding quite actively. Thus it is not possible to correlate the fragility changes absolutely with the rate of haemolysis *in vivo*. Indeed, it does not seem likely on the basis of studies *in vitro* that increased osmotic fragility can be due to the direct effect of antibodies on the cell surfaces. Nevertheless, there is significant experimental evidence that the administration of anti-red cell antibodies *in vivo* results in spherocytosis and a marked increase in fragility when the blood is subsequently tested *in vitro* (Dameshek and Schwartz, 1938; Young, Ervin, and Yuile, 1949). In man, too, the transfusion of anti-A (or anti-B) iso-antibodies to group A (or B) recipients results in an increase in osmotic fragility (Ebert and Emerson, 1946; Ervin and Young, 1950). Possibly the change towards spherocytosis is brought about as the result of stasis of blood in small vessels due to auto-agglutination (Ham and Castle, 1940). Spherocytes certainly have a greatly reduced life span, at least when the spleen is present, and marked changes always seem to be associated with a serious rate of haemolysis *in vivo*, as in Case 2 of this series who suffered from a terminal haemoglobinuria. This patient's blood underwent rapid haemolysis *in vitro* when incubated at 37° C. This was

* Both these patients are in good clinical remission eight months after splenectomy. The antibody titres have remained unaltered.

apparently due to the spontaneous lysis of extremely spherocytic red cells, for no haemolytic antibodies could be demonstrated in his serum.

Amongst other possible significant factors affecting the rate of haemolysis *in vivo* are variations in the sensitivity of the patient's corpuscles to the auto-antibodies he develops—at present of quite unknown importance—and variations in the activity of serum complement. The potential importance of complement activity in complement-haemolysin haemolytic systems like those under discussion is obvious; indeed, the results of tests for haemolysis *in vitro* were negative with the sera of Cases 11 and 17 unless additional fresh serum was first added to the patients' serum. (In these cases the complement content of the patients' fresh sera was notably diminished, as it was also in the serum of Case 12.) It is possible that these observations *in vitro* may be relevant to haemolysis *in vivo*.

Antibody Patterns in Relation to the Haemolytic Syndromes.—Although it is convenient and in most cases quite easy to classify patients suffering from haemolytic anaemias associated with the formation of auto-antibodies into separate groups on clinical grounds, as has been done with the patients separated into Groups I, II, and III of the present series, classification based on pathology is far less clear. The types of antibody present are to some extent shared amongst the different groups, and whilst cold antibodies predominate in Groups II and III, and warm antibodies in Group I, there is a considerable overlap.

It seems likely that the clinical pattern of the disease is determined at least in part by the nature of the antibodies which are being produced, whether they are of the warm or cold variety and whether cold agglutinins are present at very high titres. The minor variations within the class of antibodies which are revealed by their differing ability to act on different types of sensitive cells appear to be additional reflections of the individuality of the patients, who all react slightly differently to the usually unknown stimulus which results in the development of non-specific antibodies active against their red blood cells and the clinical syndromes of acquired haemolytic anaemia.

Summary

A series of patients suffering from acquired haemolytic anaemia has been investigated with particular reference to the presence in their sera of abnormal antibodies active against red blood cells. Abnormal non-specific antibodies were demonstrated in 19 patients suffering from idiopathic acquired haemolytic anaemia, haemolytic anaemia following virus pneumonia, or chronic haemolytic anaemia with haemoglobinuria and Raynaud's phenomena.

Trypsinized normal red cells and red cells from patients with paroxysmal nocturnal haemoglobinuria (P.N.H. red cells) were found to be valuable reagents for the detection of haemolytic antibodies; by their use non-specific haemolysins of the "warm" or "cold" variety were demonstrated in the sera of 13 out of the 19 patients.

The variations which were observed in the antibody patterns in the patients' sera are emphasized, the differences being thought to be reflections of the individuality of the responses of the patients to the stimuli causing the formation of auto-antibodies.

Haemolytic antibodies are believed to play a part in the production of haemolysis *in vivo*, but it is not claimed that they are the only cause. Other types of antibody

are probably important in this respect, and the spleen certainly contributes to the haemolytic process in many cases. In three patients belonging to Group I of the present series in whom splenectomy was carried out, a marked immediate clinical improvement was not accompanied by significant reductions in antibody titre. In these patients, therefore, the spleen seems to have been acting by removing altered or damaged red cells, rather than by being an important source of antibody.

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THE BLEEDING TIME IN NORMAL AND ABNORMAL SUBJECTS

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The bleeding time is the time between the infliction of a small standard cut in the skin and the moment when bleeding stops. At first sight it would appear that if a subject bleeds excessively from a small cut, he is likely to bleed excessively at operation, and conversely that if a subject bleeds normally from one cut he might be expected to bleed normally from another. But in neither case does the corollary always follow. These occasional failures have led to considerable caution being used in the interpretation of a bleeding time. A further reason for a critical attitude is the comparatively wide and varying limits of normal for this test given in the standard textbooks. This rather unsatisfactory state of affairs has led me to consider the possible causes of the occasional unreliability of this procedure ; it also seemed wise to reinvestigate the variation of the bleeding time in the normal population, and to compare these results with those obtained from patients who were known to have had some type of haemorrhagic incident.

Review of the Literature

Duke (1910) was the first to make a small incision in the ear and measure the time during which bleeding continued, but he realized that the information derived from this measurement had to be interpreted with care, as he reported that haemophiliacs have normal bleeding times. Ivy, Shapiro, and Melnick (1935) and Ivy, Nelson, and Bucher (1940) were the first to measure accurately the range of normal in man. They appreciated the variation of different incisions in one individual and the importance of capillary contraction in the haemostatic mechanism. In order to test the efficiency of this mechanism more severely, they put a sphygmomanometer cuff on the upper arm inflated to 40 mm. of mercury, and took the mean of the bleeding times obtained from three incisions made on the volar aspect of the forearm. They found that the range of the mean time was 0 to 2.1 minutes. Copley and Lalich (1942) tried to control as many of the variable conditions as possible. They made an incision 6 mm. deep into the pulp of the finger, and put the finger, held on a level with the manubrium sterni, into a saline bath warmed to 37° C. It is not surprising with this deep incision that the range of their results of a single incision was 0.18 to 5.36 minutes. Dishoeck and Jongkees (1940) considered that the adhesion of the lips of the incision might prevent the escape of blood ; they proposed cutting out a saucer-shaped piece of skin and pressing a thin metal plate with a small circular hole in it against the ear so that a part of

the skin protruded through the hole when it was cut off by a razor slid along the metal plate. They did not give the range of values they observed, but stated that the mean time was 3.24 minutes. The last of the more important normal series in the literature is by Pauwen, Roskam, Derouaux, and Puissant (1942), who based their work on many years of careful and extensive measurements on animals. For humans they recommended that five incisions be made round the lobe of the ear. The mean of these five times—*le temps de saignement moyen*, 0.42 to 4.42 minutes—agreed well with the mean time from the other ear, but they stressed the observation that differences between separate bleeding times in one individual might be considerable, and they also recorded wider variation in the times and a longer mean time for women, and also a slight variation with the age of the subject.

Theory of Bleeding Time Techniques

There appear to be so many subsidiary factors that may influence the result of the estimation of bleeding time that, quite apart from what may be described as the essential haemostatic mechanism, this type of investigation may quite easily give a misleading answer. The time recorded is the longest time during which any of the incised capillaries continues to bleed. If there are variations between individual capillaries, obviously the more capillaries that are cut, the higher the chances of cutting one or more that bleed for a long time.

The number of capillaries coming to the surface differs in different parts of the body, so the site of the incision will decide the number of capillaries cut. Furthermore, it has proved impossible to standardize accurately the depth of these experimental incisions, so on occasions arterioles and venules must also be cut. However sharp the cutting instrument may be, it must to some extent tear its way through the tissues, a process that on a larger scale often causes almost bloodless lacerations; it appears, therefore, that the sharpness of the instrument will influence the result of this investigation. Once blood has escaped from a blood vessel it has to get out on to the surface of the skin. The elasticity of the skin usually pulls the lips of a linear incision apart, but in old people with inelastic skin the blood may not be able to escape and a small subcutaneous haematoma is formed, and little blood appears on the surface. There are in addition a number of other experimental conditions which may influence the result, such as raising or lowering the venous and capillary blood pressure, the temperature of the skin, and whether it is immersed in a fluid to eliminate the effect of drying.

Lastly, there is the almost philosophical problem of how the normal is defined. The majority of most biometric measurements fall fairly close together, but there are often a few subjects who are within normal limits by all criteria, except that they give a result to this one test that is appreciably different from the majority. Should the normal range extend to include these few measurements, or are these few to be regarded as "abnormal?" To quote Ryle (1947):

> Variability is one of the most distinctive and necessary attributes of life, which admits of no constant and no norm.

Experiments Using the Ivy Technique

In spite of its apparent theoretical disadvantages, it was necessary to find out if in fact this type of investigation could indicate the presence of a haemorrhagic diathesis. A small scale trial of several methods was first carried out including the routine Ivy tech-

nique, the result of warming the skin before making an incision, the effect of different types of incision, and the ear-saline technique (Macfarlane, 1946) which consists of pricking the lowest part of the ear and immersing the dependent part in a glass cylinder of saline at 37° C. so that the blood falls in a thin stream through the saline and the moment when bleeding stops can be accurately read. Inspection of these results showed slight variations between techniques, but even larger differences within each technique, and sometimes large differences in one individual when one type of test was repeated. One apparently normal person continued to bleed for over 15 minutes.

When all these methods were reviewed it became clear that none of them combined painlessness and ease of performance with the advantage of controlling all the subsidiary factors that might effect the bleeding time. For the main series of observations on "normal" people I decided to use the ordinary Ivy technique, partly because it has as many theoretical advantages as any other method except that of Dishoeck and Jongkees, and also because it is one of the methods in general use in this country.

Method.—It was decided to make several estimations of the bleeding time by the chosen method in a number of apparently healthy people with two primary objects in view: first, to observe the variation of the bleeding time in the same individual and from one person to another; and second, to collect information about, or measure, as many different factors as possible that might influence the bleeding time. It was hoped thereby to be able to estimate from a survey of the whole series the influence, if any, of each of these incidental factors. Every volunteer was accepted, and his or her results included in the series, until a sufficient and approximately equal number of observations had been made on people of all ages and both sexes. A number of people with red hair were specially chosen, because it has been claimed that red-headed people bleed longer than others.

All the subjects accepted were in good general health. Some were laboratory technicians, nurses, labourers, and others were patients who were asked to volunteer so that the possible effect of rest in bed might be investigated. Only those patients with purely local lesions were accepted. The majority had herniae and were tested before operation; others had local gynaecological abnormalities, or were awaiting plastic surgical treatment.

At the beginning of the interview with each subject a record was made of the age, sex, physical build, hair colour, skin thickness and elasticityv, and his opinion as to whether he bled abnormally from cuts or bruised more than other people. In case there were day-to-day variations in the same individual, each subject was visited twice, usually on consecutive days, to get paired observations. On each occasion a record was made of the time of day, hours after a meal, and the blood pressure at the end of the interview. The forearm skin temperature was roughly measured by a mercury thermometer with a long cylindrical bulb covered by sorbo rubber tightly strapped on the forearm above the wrist.

For the sake of uniformity I carried out all the investigations myself and with the same apparatus. One spring lancet with a blade 2 mm. wide, 0.5 mm. thick, and set at 2.5 mm. depth was used throughout, but it had to be sharpened a number of times. Three incisions were made on the volar aspect of one arm at the first visit, while the other arm was used at the second visit with a manometer cuff on the upper arm inflated to 40 ± 5 mm. Hg. The incisions were made at five-second intervals. Then each was lightly "blotted" every 15 seconds by sliding the edge of a piece of filter paper slowly over the cut, a separate filter paper being used for each, so that a permanent record was made of the time taken, indicated by the number of blots, and a rough record of the quantity of blood lost. Since bleeding from some of the incisions decreased extremely slowly, it was found impossible to devise any exact definition of the end-

point. All incisions that continued to bleed were observed for 15 minutes. Observation was then discontinued.

Results.—The results obtained by this method and the two other methods used subsequently are tabulated below (Table I).

TABLE I

COMPARISON OF RESULTS WITH THREE TECHNIQUES

Technique	Site	No. of Incisions per Subject	No. of Subjects	Total No. of Observations	Mean Bleeding Time (minutes)	
					Average	Range
Ivy	Forearm	6	98	588	♂ 5·0 ♀ 6·5	1·8–10·0 1·3–10·5
Ear-saline ..	Ear	2	60	120	♂ 3·7 ♀ 4·7	2·0–10 0 2·0–12·0
Duke	Ear	2	67	134	♂ 4·2 ♀ 5·6	0– 6·5 0·5–10·1
Combined totals			225	842	5·06	0–12·0

♂ = Males. ♀ = Females.

The most impressive result was that this method in the present series gave isolated bleeding times from 0 up to 15+ minutes, i.e., blood was flowing freely at the end of 15 minutes, whereupon the observation was discontinued. For the statistical analysis and for the purpose of working out the mean bleeding times these 15+ minute readings have been taken as 15 minutes exactly ; this is obviously incorrect, but no fairer way of analysing these results could be devised. As far as can be judged, the effect of this assumption is likely to be small, and would only make interpretation difficult in borderline cases. In the ensuing analysis the findings are all either definitely significant, or quite insignificant, so that this technical point is fortunately unimportant. There was so much variation in the results within individuals that it was apparent that only statistical analysis would reveal any slight differences within the group of observations as a whole.

Fig. 1* shows the general pattern of distribution, with a fairly large number of observations of 0 minutes (i.e., no bleeding observed) even though a reasonable incision had been made. The histogram shows that there is a high peak of frequency of observations between three and six minutes and that there is a very long tail to the right where the curve appears to become asymptotic: in other words, a few subjects had some very long bleeding times. Fig. 2, in which the means of all six readings for each subject are recorded, shows much the same distribution except that there is not such a wide scatter along the time scale, which signifies that the very long and the very short bleeding times were cancelled out by intermediate results.

Inspection of the results shows that females tend to have longer bleeding times than males. This observation is supported by the difference in the mean bleeding

* In all the histograms where an arrow is used it indicates the dividing line between 95% of the observations and the 5% of observations which had the longest times.

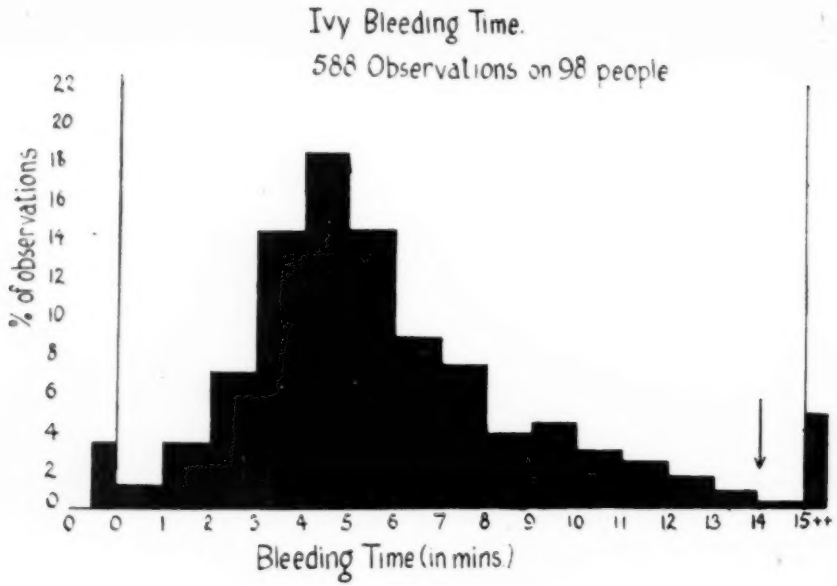
Ivy Bleeding Time.
588 Observations on 98 people
% of observations
22
20
18
16
14
12
10
8
6
4
2
0
0 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15++
Bleeding Time (in mins.)

FIG. 1

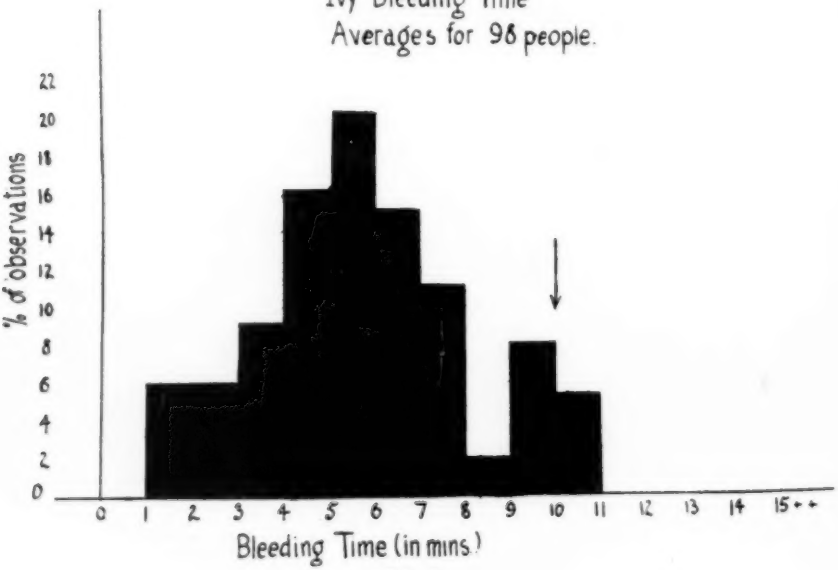
Ivy Bleeding Time
Averages for 98 people.
% of observations
22
20
18
16
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15++
Bleeding Time (in mins.)

FIG. 2

time for all males compared with that for all females (Table I and Fig. 3). Any further differences were obscured by the wide variation within the individual so results were submitted to the usual statistical analysis of variance (Table II).* The preliminary analysis shows that there is a significantly greater degree of variation of the bleeding times between different subjects when compared with the variation within the individual subjects ; in other words there is more variation between results on different subjects than occurs between readings on the same subject. Because of the marked sex difference all future analyses have been divided into two, one for each sex.

The second analysis carries the procedure of the first into more detail by subdivision of the two basic comparisons. First, the general comparison between patients may be regarded as composed of three distinct elements: between sexes, between male patients, and between female patients. Separate variance estimates can be derived from each of these three comparisons. Similarly it is possible to subdivide the general comparison " within patients." Three of the readings on each patient were taken at one time, three at another. Thus for each sex separately one variance estimate may be derived from a comparison of the mean bleeding times

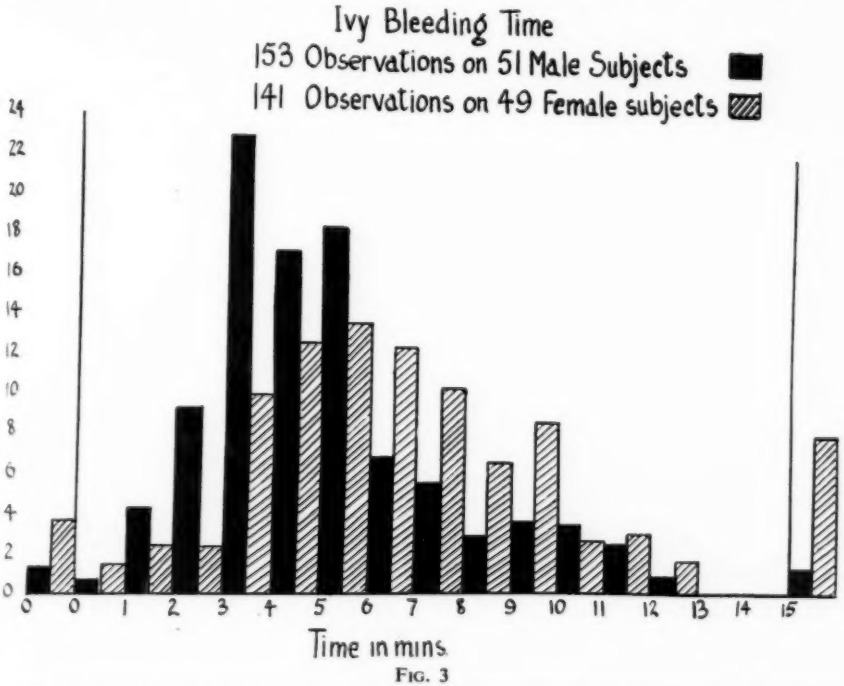

FIG. 3

* The statistical analysis was supervised and largely carried out by Mr. I. Sutherland, of the Nuffield Department of Social Medicine.

TABLE II

ANALYSES OF VARIANCE

	Difference	S.S.	Variance Estimate	Variance Ratio
Preliminary analysis				
Between patients	97	3,388·3802	34·932	4·67**
Within patients	490	3,663·9479	7·4774	
Total	587	7,052·3281		
Detailed analysis				
Between sexes	1	342·6120	342·61	
Males:				
Between patients	50	938·8741	18·777	3·34**
Between visits to same patient ..	51	540·8333	10·605	1·87**
Within visits	204	1,145·3334	5·6144	
Females:				
Between patients	46	2,106·8941	45·802	7·83
Between visits to same patient ..	47	878·2188	18·686	3·19
Within visits	188	1,099·5624	5·8487	
Total	587	7,052·3281		

Variance ratios: (*a*) Sex difference:

$$\frac{342{\cdot}61}{18{\cdot}777} = 18{\cdot}25^{**}; \qquad \frac{342{\cdot}61}{45{\cdot}802} = 7{\cdot}48^{**}$$

(*b*) Males:

$$\frac{18{\cdot}777}{10{\cdot}605} = 1{\cdot}77^{*}; \qquad \frac{10{\cdot}605}{5{\cdot}614} = 1{\cdot}89^{**}$$

(*c*) Females:

$$\frac{45{\cdot}802}{18{\cdot}686} = 2{\cdot}45^{**}; \qquad \frac{18{\cdot}686}{5{\cdot}8487} = 3{\cdot}19^{**}$$

	Difference	S.S.	Variance Estimated	Variance Ratio
Linear Regression on Age				
Males:				
Linear regression	1	45·9643	45·964	2·52
Other differences between patients ..	49	892·9098	18·223	
Females:				
Linear regression	1	13·8106	13·811	1 : 3·37
Other differences between patients ..	45	2,093·0835	46·513	

of the same patient on two different occasions, and another from the variation in readings on the same patient at the same time. This leads to four more estimates, giving a total of seven in the detailed analysis. Any two of these estimates may be compared.

The findings may be summarized as follows. (1) There is a significant sex-difference in bleeding time ; females have a longer bleeding time at all ages. (2) The

subject's bleeding time varies significantly from time to time. (3) There are "constitutional" differences between patients which are not obscured by the "temporal" variation just mentioned. (4) There is no evidence that the constitutional differences are connected with age, hair-colour, physical constitution, skin thickness, skin elasticity, or skin temperature.

Series Using the Ear-Saline Technique

The results obtained by the Ivy technique do not agree with those of Ivy's own series, and some remarkably long bleeding times were recorded, due presumably in part to the fact that I must have made a larger incision. It was accordingly decided to measure the bleeding times of healthy people obtained by two other techniques. The second method was that suggested by Macfarlane (1946). I thought it wise to use a different site, and the control of temperature and drying appeared to be the advantages of this method. For the third series I chose the Duke method (1910), which is perhaps the most commonly used.

The method for the ear-saline technique has already been described and in this series I made a single incision in one ear and measured the bleeding time and immediately repeated the procedure on the other ear. No questionary was filled in, the sex and age being the only facts noted.

Results.—A histogram (Fig. 4) of the individual observations is essentially similar to those obtained by the Ivy technique, but it will be seen that no cuts failed to bleed, while three out of 120 incisions bled for over 15 minutes. A higher percentage of observations falls close to the mean, which is considerably shorter than with the Ivy technique (Table I) and 66.5% of the readings fall between one and five minutes.

Statistical analysis confirms that there is a significantly greater variation between patients compared with the variation between the two ears, and the sex difference is maintained.

Series Using the Duke Technique

For this series the puncture made by the spring lancet was modified, in accordance with Duke's original suggestion that an incision be made of sufficient size to produce blots on the blotting paper about 1 cm. in diameter.

Method.—The cleaned ear was lightly pricked on the anterior surface of the lobe and the issuing blood blotted every half minute from the bottom of the lobe, so that the wound was never disturbed. The second ear was pricked 15 seconds later, and the two bleeding times were estimated simultaneously. The moment at which bleeding stopped was more difficult to determine than in either of the two other methods.

Results.—A histogram (Fig. 5) of the individual results shows a fairly large number of zero readings. These were because the wound did not bleed, and not because it could not bleed, since it was confirmed that most of these incisions would bleed if the skin was gently stretched. It will be noted that no single observation continued for more than twelve and a half minutes. It is not known whether this was due to the smallness of the sample examined, to the slightly smaller puncture, or to other differences in technique. Essentially, however, this curve is similar to the others.

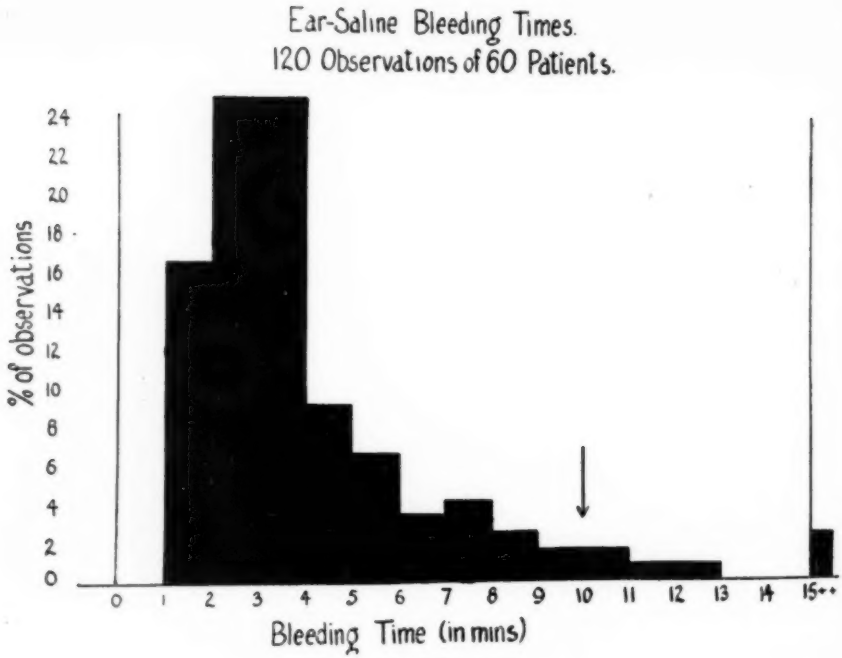
Ear-Saline Bleeding Times.
120 Observations of 60 Patients.
% of observations
0
2
4
6
8
10
12
14
16
18
20
22
24
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15++
Bleeding Time (in mins)

FIG. 4

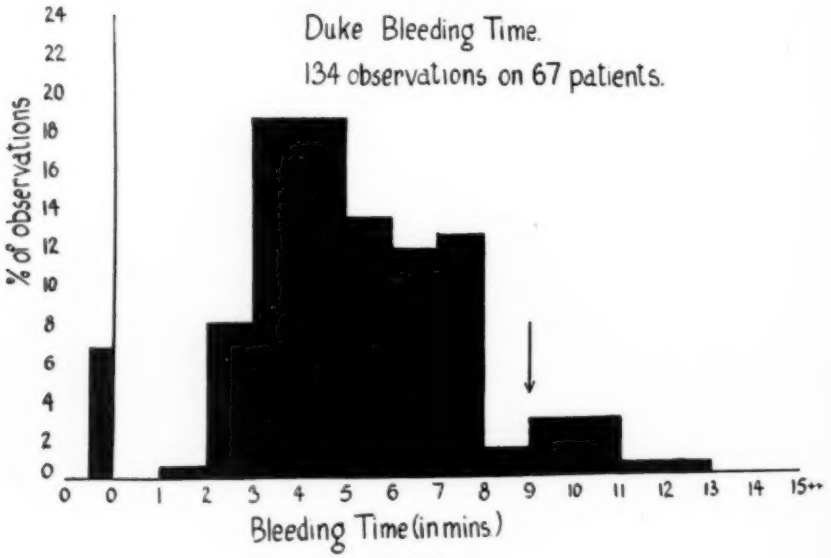
Duke Bleeding Time.
134 observations on 67 patients.
% of observations
0
2
4
6
8
10
12
14
16
18
20
22
24
0 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15++
Bleeding Time (in mins.)

FIG. 5

The Three Techniques Compared

A composite histogram (Fig. 6) of the three techniques shows a very similar distribution of results. The mean time is shortest by the ear-saline time, and longest by the Ivy technique ; the long tail to the right is obvious in each series.

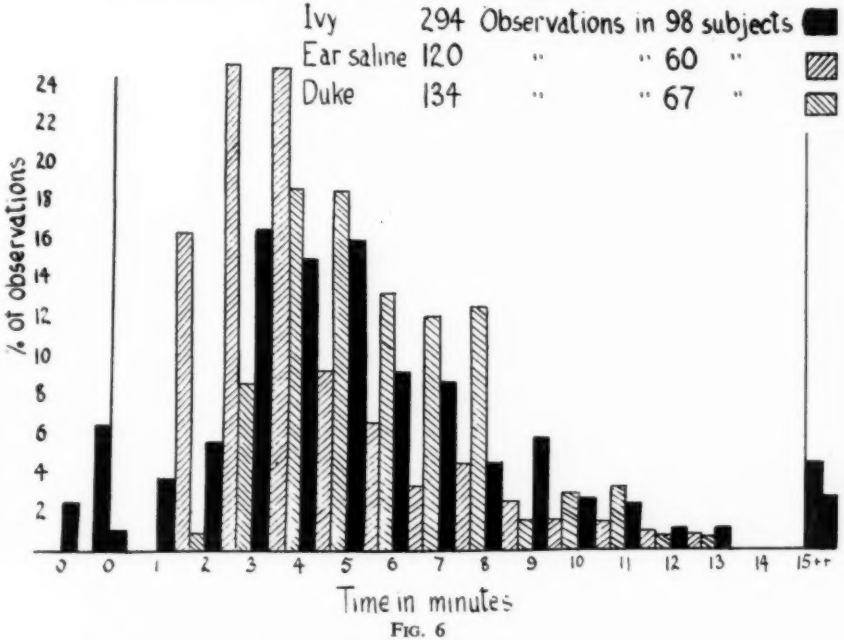

FIG. 6

Nine male and nine female subjects had volunteered for all three series, and these results were tabulated and subjected to statistical analysis. Again there is significantly greater variation between patients and between different visits to the same patient than there is variation within patients at one visit. There is no significant variation between techniques that cannot be accounted for by the two factors just mentioned.

The Bleeding Time of Abnormal Patients

The results of all the bleeding times carried out as part of the routine of the Pathological Laboratory of the Radcliffe Infirmary during two years will now be reported. These results were obtained by a number of technicians using different instruments, but they all used the Ivy technique and usually made three incisions. For most of these patients the laconic diagnosis on the pathological request form was accepted ; in a number of patients, particularly those with long bleeding times, the diagnosis was confirmed from the case notes. In all cases, however (even in those with no diagnosis given on the request form), it may confidently be assumed

that the doctor who asked for the investigation thought that there was or might be something wrong with the patient's haemostatic mechanism.

The results of 278 bleeding times, almost all of which are the mean of three observations, were available for study. They were arbitrarily divided into two groups, first, those from patients with more than 100,000 platelets per ml., and secondly those with severe thrombocytopenia and one case of congenital athrombocytopenic purpura. Two hundred and five bleeding times were available from patients with many platelets, and the diagnoses are summarized in Table III.

TABLE III

DIAGNOSIS OF PATIENTS WITH MORE THAN 100,000 PLATELETS PER ML.

Diagnosis	No. of Observations
Recovered thrombocytopenic purpura	26
Purpura present; normal platelets	25
Epistaxis	5
Haemorrhage, post-operative or in history	33
Bruising	11
Menorrhagia, etc.	30
Haemophilia	5
Chronic arthritis on gold treatment	11
Miscellaneous	38
No diagnosis given	21
Total	205

Each group of results was examined separately and did not differ significantly from the others so that the whole 205 results can be represented in the form of a histogram (Fig. 7). It will be seen that this histogram is of exactly the same shape as Figs. 1 and 2 of the 98 normal subjects. The mean for the abnormal patients is 4.46 minutes, and that for normal males and females by the Ivy technique is 5.8 minutes. Since these two histograms are for all practical purposes identical, it is clear that this test does not help to reveal those patients with more than 100,000 platelets per ml. of blood, who bleed excessively at operation or have other haemorrhagic manifestations. In view of the similarity of the histograms of the normal results obtained by the ear-saline and the Duke methods with those obtained by the Ivy method, it is also reasonable to assume that neither of these two methods and probably any of the other recognized methods would have revealed any abnormality in these 205 observations. Furthermore the similarity of these two histograms (Figs. 7 and 2) indicates that it was not some personal variation of the technique which produced the long times, since many technicians carried out the tests on the abnormal patients. It seems therefore that the error of the method due to observer differences are quite insignificant when compared with the variation of the subjects.

The only Radcliffe Infirmary results excluded from consideration above are those from patients with a platelet count below 100,000 per ml. and the one case of athrombocytopenic purpura (Macfarlane, 1941). The diagnoses and numbers of patients and observations are listed in Table IV.

Although the numbers in each group are small, the bleeding times were fairly uniformly long, and I think it is justifiable to draw a histogram of this group as a

whole (Fig. 8). It will be seen that in addition to 39.7% of the results continuing for 15 minutes or over, the rest show many more prolonged times (9–14 minutes) than are recorded in the normal population. These last results are, of course, nothing new; it has long been known that thrombocytopenic patients bleed excessively and have a grossly prolonged bleeding time.

TABLE IV

Diagnosis	Platelets/ml.	Patients	Observations
Essential thrombocytopenic purpura ..	<100,000	18	59
Leukaemia .. .. Chronic nephritis .. .. Cirrhosis .. .. Myelosclerosis	<100,000	8	10
Athrombocytopenic purpura	>100,000	1	4
Totals		27	73

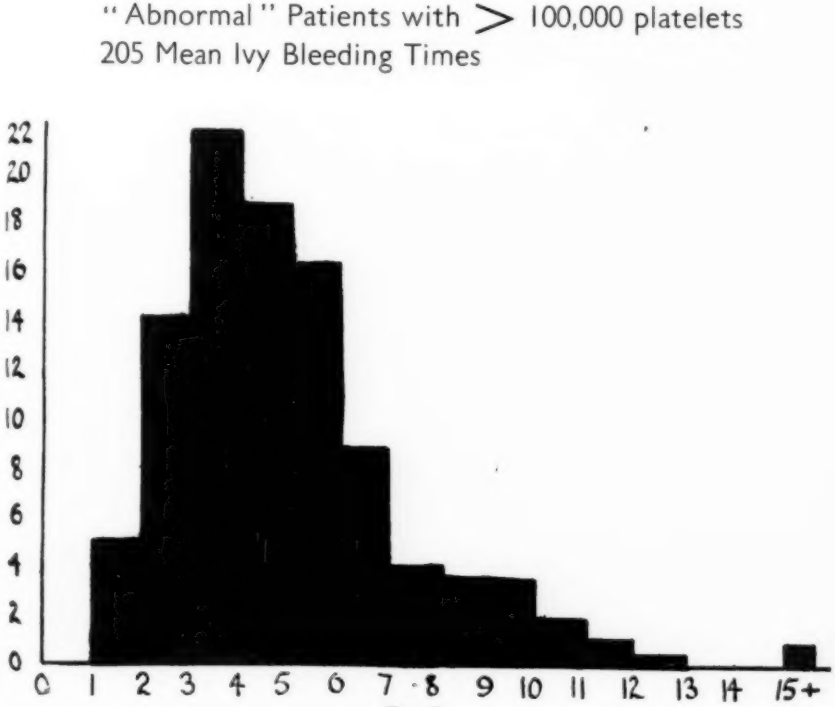

FIG. 7

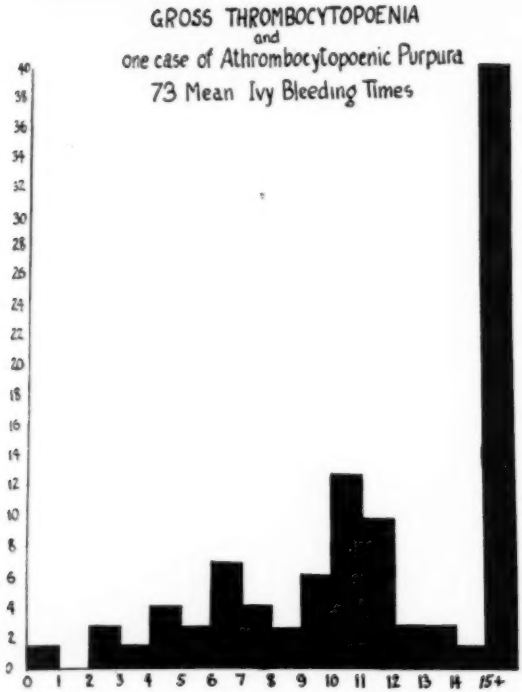

FIG. 8

The only patient that must be singled out is the girl with athrombocytopenic purpura who usually had a very long bleeding time and always had normal platelets. It might be argued that this was the only case in which the bleeding time had given information that could not be obtained from counting the platelets. This is true, but in patients like this, and this girl was no exception, there is usually such a definite history of haemorrhagic episodes, that even without a bleeding time, an abnormality of the haemostatic mechanism must be suspected which may be confirmed by estimating the bleeding time. Repeated estimations of the bleeding time are, however, essential in finding minor degrees of this syndrome in members of an afflicted family, who do not have a definite history of bleeding.

Conclusions

The surgeon has long been asking for a test which will reveal those cases which will bleed excessively at operation, and the physician will often ask for laboratory tests to support a history of easy bruising, purpura, or excessive bleeding from cuts. The results of estimating the bleeding time of all such cases referred to the Pathological Department of the Radcliffe Infirmary during the past two years give no

more evidence than is obtained from a single platelet count. (Although the error of any method of counting the platelets is large, the critical level of about 100,000 per ml. is so far below the normal level—roughly 300,000 by the method used—that confusion is not likely to arise from the inaccuracies of this test.) If the platelet count is normal, then it can safely be assumed that the bleeding time also will be " normal " ; that is to say it will fall probably between two and six minutes, but may be much longer although it will not repeatedly continue for more than 15 minutes. The only exceptions to this rule are the rare capillary abnormalities like athrombocytopenic purpura, the Ehlers-Danlos syndrome, and congenital telangiectasia, in which syndromes the platelets are present in normal numbers, but the bleeding time may be grossly prolonged if the abnormal capillaries are punctured. As a rule patients with these syndromes give a characteristic history.

We partially understand a few of the component parts of the haemostatic mechanism, but we have so little understanding of the process as a whole that we can only speculate about the cause of the gross variations encountered when estimating the bleeding time in apparently healthy people.

Summary

Three estimations of the Ivy bleeding time were made on 98 apparently healthy volunteers on two separate occasions. The individual times varied from zero to more than 15 minutes, while the majority of the recorded times were between three and six minutes. Two further series by the Duke method (67 subjects) and the ear-saline method (60 subjects) confirmed these results with only minor differences.

A study was made of the results from all the patients referred to the Pathological Department of the Radcliffe Infirmary, Oxford, during the last two years, who complained of haemorrhagic manifestations. These results were divided into those from patients with over of 100,000 platelets per ml. of blood and, those with gross thrombocytopenia. The bleeding times of the patients with over 100,000 platelets per ml. were indistinguishable from those of the normal controls ; the majority of the patients with gross thrombocytopenia had long bleeding times. It is concluded that, except in a few rare diseases, the bleeding time gives no more information than a platelet count.

I should like to acknowledge the great help Dr. R. G. MacFarlane has given me at all stages of this work, without whose encouragement this paper would never have been written ; I also have to thank Mr. I. Sutherland, of the Nuffield Department of Social Medicine, for the analysis of variance, and the volunteers for their patience and longsuffering.

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J. clin. Path. (1951), **4**, 286.

HYPERHAEMOLYSIS DURING THE PREMENSTRUAL PERIOD

(PRELIMINARY REPORT)

BY

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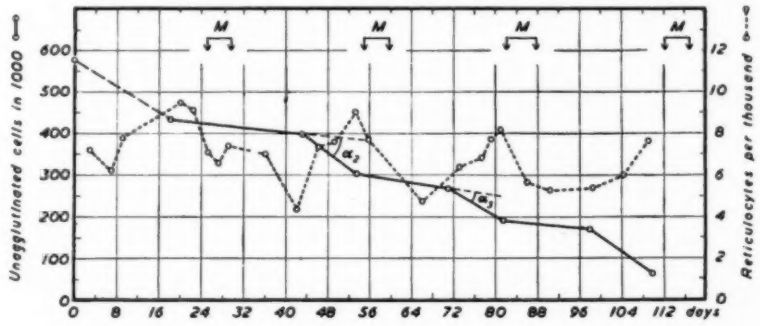
From the Medical Clinic, University Hospital, Uppsala, Sweden

(RECEIVED FOR PUBLICATION SEPTEMBER 25, 1950)

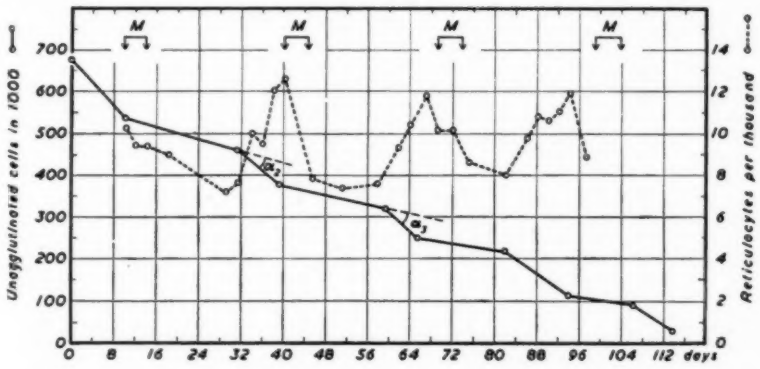
Winifred Ashby (1921), in her transfusion studies by means of differential agglutination, claimed that there was a definite periodicity in the elimination of transfused red cells in women in connexion with the menstrual flow. She suggested that this phenomenon was probably due to hormonal influences, but she gave no further explanation of these irregularities as a whole. Several later workers, using the same method, have failed to show this periodicity (Wearn, Warren, and Ames, 1922), but they based their conclusions on only two patients who were in the menopausal period. Since that time, the reports on this subject have been very scarce, only one paper (Callender, Powell, and Witts, 1947) dealing with this problem in any detail. These investigators followed the survival of transfused red cells in the circulation of four normal women and found that the cells disappear discontinuously and that the blood loss in women exceeds that in men by 400 ml. per month. It is not clear, however, from their curves exactly where, on the elimination curve, the cell disappearance is most pronounced ; it is only apparent that it may occur in connexion with menstruation. The data obtained in their cases are relatively few, and it is thus impossible to analyse the detailed relationships in these respects. The authors emphasize that the actual blood loss during the menstrual period is much more than can be expected from a normal menstruation.

In this study the differential agglutination method was used to study the survival of red cells in one 5-year-old girl, five normal young women, and two women over 60 years of age. The girl showed a disappearance curve of the same linear type as that found in boys and men. This type of curve was also obtained in the two elderly women. The survival curves in the young women, on the other hand, differed in a significant manner from those just mentioned. In accordance with earlier observers a more rapid drop in the curve was found in connexion with menstruation, but the drop begins about ten to six days before menstruation and goes on until the period begins or, in some instances, one or two days earlier. This premenstrual change appears simultaneously with the progesterone phase of the menstrual cycle, i.e., parallel with the well-known premenstrual increase in body temperature. These changes could be due to a simple dilution of the blood at this time, but in this case the curve would then revert to the extrapolated

elimination curve before the drop began. This is not the case, however; at the beginning of the menstrual bleeding the curve bends more horizontally but is still falling. The fact that there is no change in the total plasma protein (Van Slyke method) during this period also makes a premenstrual dilution

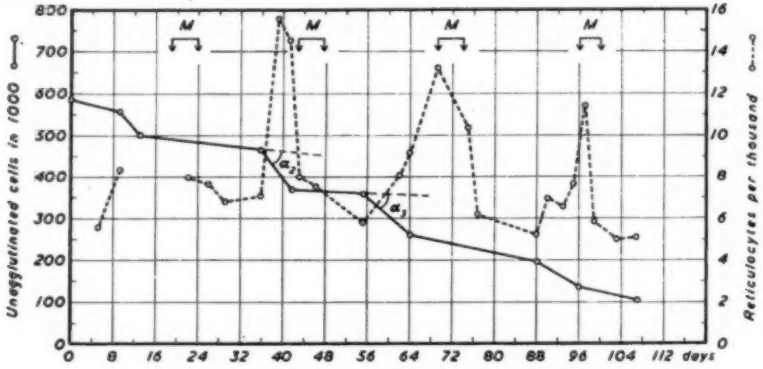


of the blood unlikely. The simplest explanation seems to be that there is an increased blood destruction during the progesterone phase of the cycle, and this could explain the excessive blood corpuscle disappearance in women beyond the actual flow during menstruation. This belief receives strong support from the fact that there is a considerable reticulocytosis, beginning about ten days before menstruation and increasing day by day until the start of menstruation



(10,000 cells counted on every slide; error 5–8%). On that day there is a rapid drop in the reticulocyte count, which remains within normal limits in spite of the menstrual blood loss. This view is partially in opposition to the observation made by C. M. Plum (1942) and R. Plum (1947) in their work on the reticulocyte ripening factor, where they claim that there is a reticulocyte increase due to menstruation but that the peak occurs one or two days after the onset

of menstruation. In the graphs here shown all curves are smoothed according to successive means, and the original observations are based on determinations made every third day during the first half of the menstrual cycle and every second day during the premenstrual period of unagglutinated cells remaining after differential agglutination.



By a closer analysis of the survival curve in these menstruating women it can be observed that the curve has a flatter course from menstruation to the beginning of the progesterone phase, thus indicating a less rapid destruction during this period, corresponding to a considerably longer survival time than normal. The normal survival time is about 120 days, and the daily destruction of red cells is calculated to be about 0.83% in a normal man. Table I shows the different values

TABLE I

CALCULATED DAILY RED CELL DESTRUCTION VALUES IN THREE CASES

	Daily Red Cell Destruction (%) during Premenstrual Period	Mean
Case 1—α_2	1.56	1.42
α_3	1.28	
Case 2—α_2	1.56	1.55
α_3	1.73	
Case 3—α_2	2.77	2.36
α_3	1.96	

obtained for the calculated daily destruction of R.B.C. in three women in the two middle premenstrual periods, these two being probably the most reliable ones. The two other women show similar values. It is of special interest to point out that the angle α, between the two parts of the curve in each period, seems to be correlated to the height of the reticulocyte peak, i.e., the greater the angle the greater the haemolysis, therefore the more pronounced is the reticulocyte response.

These observations indicate the presence of a considerably increased blood destruction during the last ten to six days before the beginning of normal menstruation and explain the findings of earlier workers of an increased blood loss during the menstrual period. The nature of this hyperhaemolysis is obscure, but it is probably due to hormonal influences. The spleen plays no part in this haemolysis, as a similar curve has been obtained in a woman who had undergone splenectomy.

Further experiments on the elimination curve in young oophorectomized women before and after progesterone treatment are in progress.

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THE LUPUS ERYTHEMATOSUS CELL INCLUSION PHENOMENON

BY

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From the Department of Pathology, Postgraduate Medical School of London

(RECEIVED FOR PUBLICATION JANUARY 11, 1951)

In 1948 Hargraves and his associates at the Mayo Clinic described two new entities in marrow biopsy material, the " tart " cell and the " lupus erythematosus " (L.E.) cell. Since then the morphology and the mechanism of production of these " cells " have been intensively studied in the United States. It is the purpose of this communication to report the finding of L.E. cells in the bone marrow films of four cases of acute disseminated lupus erythematosus.

Morphology

Lupus erythematosus cells are cells containing a homogeneous mass of apparently phagocytosed material which stains purple with Romanowsky dyes. " Tart " cells are similar cells in which the inclusion body is not structureless but retains a recognizable chromatin pattern.

Usually, but not always, it is possible to recognize the type of host cell. Most of them are neutrophil polymorphonuclears or neutrophil band forms (Figs. 1, 4, and 6); very rarely eosinophils and monocytes are affected (Fig. 2). Myelocytes, promyelocytes, myeloblasts, erythroblasts, plasma cells, and lymphocytes have not been observed to contain these bodies. The affected cells are often found in clumps of three or four; occasionally larger clumps of 10 to 20 cells are seen (Fig. 3). These clumps are often associated with free, extracellular purple-staining masses morphologically and tinctorially identical with the inclusion bodies. Quite commonly and characteristically two or more cells are seen sharing one " body " (Fig. 4), and less commonly two " bodies " are found in one cell (Fig. 5).

Methods for the Demonstration of Lupus Erythematosus Cells

Lupus erythematosus cells cannot be found in films made at the time the marrow is aspirated. This has been emphasized by Dameshek and Bloom (1950), Berman, Axelrod, Goodman, and McClaughry (1950), Hargraves (1949), and Haserick and Bortz (1949), but is still insufficiently appreciated. This has also been the case in all four of the writer's patients. Lupus erythematosus cells were found in moderate numbers in citrated marrow preparations, prepared as described below, but careful observations on the corresponding direct marrow films failed to show even one L.E. cell. It is imperative, therefore, that some anticoagulant be used. The nature of the anticoagulant seems to be of small importance. Berman *et al.* (1950) and Haserick (1950b) used heparin (0.1 mg./ml. of fluid); Heller and Paul's oxalate mixture as recommended by Wintrobe (1946) is also suitable.

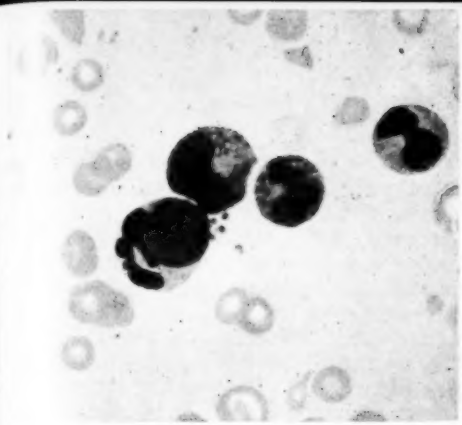

FIG. 1.—Neutrophil containing inclusion body. Citrated marrow film × 800.

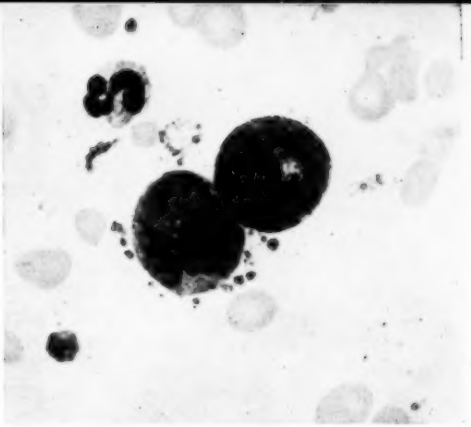

FIG. 2.—Monocyte containing inclusion body. Citrated marrow film × 800.

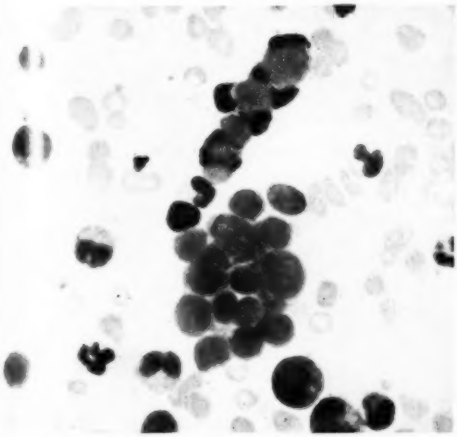

FIG. 3.—Clump of free masses and L.E. cells. Citrated marrow film × 490.

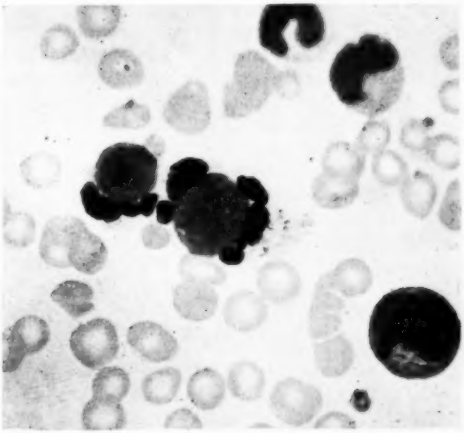

FIG. 4.—Two neutrophils surrounding one inclusion body. Citrated marrow film × 800.

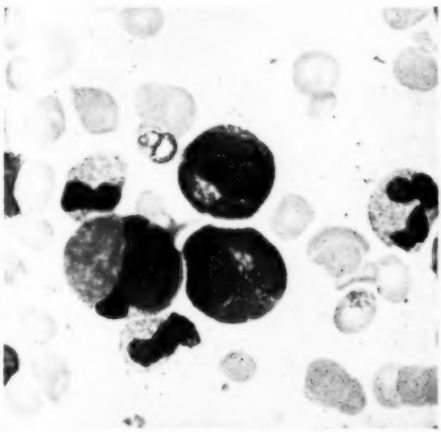

FIG. 5.—Two inclusion bodies in one cell. Citrated marrow film × 800.

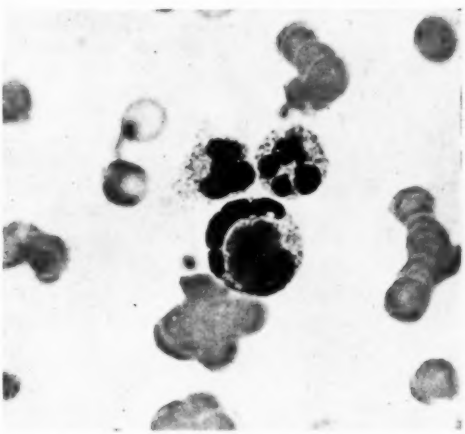

FIG. 6.—L.E. cell in peripheral blood. "Buffy coat" film from oxalated blood × 800.

Sporadic accounts of finding L.E. cells in the peripheral blood have been published (Sundberg and Lick, 1949; Hargraves, 1949; and Moffat, Barnes, and Weiss, 1950). They are said, however, to be found in too small numbers to assist in the diagnosis of acute disseminated lupus erythematosus. Nevertheless, in all four of the cases which I investigated the L.E. cells were demonstrated in appreciable numbers in the peripheral blood (Fig. 6). These preparations have proved quite as satisfactory diagnostically as the marrow preparations and have also been very useful for serial studies in attempting to assess alterations in the L.E.-producing factor in patients under treatment with A.C.T.H. This method obviates the necessity for doing repeated marrow punctures on sick patients.

Recommended Techniques

Bone Marrow.—The marrow puncture is done in the usual way. A small amount (0.2–0.3 ml.) of marrow fluid is withdrawn, placed in a small 7 × 30 mm. tube containing two drops of 3% sodium citrate and gently mixed with the anticoagulant. The suspension of marrow is removed with a Pasteur pipette, placed without further delay in a Wintrobe haematocrit tube, and centrifuged for five minutes at 2,000 r.p.m. Longer centrifuging or higher speeds are unnecessary and may pack the cells too firmly. After centrifuging, the marrow separates into four layers. At the top is a fatty layer, below this a layer of plasma, below this the nucleated cell layer (buffy coat or myeloid-erythroid layer) containing the nucleated marrow cells of both granulopoietic and erythropoietic series, and at the bottom the non-nucleated red cells. The fat and upper layer of plasma are withdrawn by means of a Pasteur pipette and discarded, leaving behind a volume of plasma equal in volume to the nucleated cell layer. The remaining plasma and as much as possible of the nucleated cell layer is then withdrawn by a Pasteur pipette as well as a millimetre or two of the underlying red cell layer. The contents of the pipette are then emptied into a waxed watch-glass and well mixed by alternately drawing up and expelling the fluid. Drops of the fluid are finally placed on clean slides and films made in the usual way. These are allowed to dry in air, then fixed and stained as for ordinary marrow films.

Sodium citrate seems to be a preferable anticoagulant to heparin or mixed oxalates, because the solution is easy to prepare, and it is stable and reliable. In addition, staining is not impaired and the morphology of the marrow cells is not affected.

Peripheral Blood.—Blood is taken in suitable quantity into Heller and Paul's oxalate mixture (six parts ammonium oxalate, four parts potassium oxalate: 2 mg. of the mixed salts per ml. of blood), gently mixed, placed in a Wintrobe tube, and centrifuged for 15 minutes at 2,000–3,000 r.p.m. Centrifuging whole blood for a shorter time is, in general, insufficient to spin down the leucocytes satisfactorily. Most of the plasma is discarded, the "buffy coat" and upper millimetre of red cells are withdrawn, mixed, spread, and stained as for the marrow preparations.

Oxalate is preferred to citrate as the anticoagulant for peripheral blood solely because it is the best to use for other routine studies, which can therefore be carried out on the same venous sample. The technique must, however, be carried out shortly after withdrawing the blood or the leucocytes will show degenerative "oxalate" changes.

Hargraves (1949) has described a special container for centrifuging blood. This seems an unnecessary refinement, as the haematocrit tube has proved quite satisfactory.

Discussion

The Role of Anticoagulants.—It is interesting to speculate why L.E. cells can only be found in blood or marrow treated with anticoagulants and never in

fresh films. As three such widely different chemical substances as heparin, sodium citrate, and potassium and ammonium oxalate mixture can be used, it is extremely unlikely that it is a direct chemical action on the cells. Furthermore, by using defibrinated blood (i.e., without any added anticoagulant) L.E. cells have been demonstrated in one case of the present series in which the method was tried. This method is not, however, recommended, as many leucocytes are necessarily lost in the process of defibrination. Lee, Michael, and Vural (1950) have also demonstrated L.E. cells in clotted blood. It seems, therefore, that the role of anticoagulant is to provide the time necessary for the plasma factor to act on the leucocytes. Failure to find L.E. cells in active cases of acute disseminated lupus erythematosus is probably due to not having used an anticoagulant (Stich, 1950).

The Plasma Factor.—It has been repeatedly shown (Haserick, 1950a ; Haserick, 1950b ; Moffat *et al.*, 1950 ; Berman *et al.*, 1950 ; Hargraves, 1949 ; and Haserick and Bortz, 1949) that the factor producing the alterations in the leucocytes is present in the plasma of patients with acute disseminated lupus erythematosus and not in the leucocytes themselves. Patient's plasma when incubated with normal human leucocytes, normal human marrow, or even animal marrow will produce typical L.E. cells. Haserick (1950a), by adding L.E. plasma to marrow and making films at 60-second intervals, demonstrated that the leucocytes clumped five or six minutes after mixing and L.E. cells appeared after 12 to 13 minutes. The plasma factor is said to maintain its potency for a long period if kept below $-20°$ C. Berman *et al.* (1950) have shown that the L.E. factor is present in the gamma-globulin plasma fraction.

Auxiliary Tests.—Various auxiliary methods have been used for demonstrating the plasma L.E. factor. Haserick (1950a, b), Haserick and Bortz (1949), Berman *et al.* (1950), and Hargraves (1949) have used L.E. plasma mixed with bone marrow from patients with various diseases, and Haserick (1950b) and Berman *et al.* (1950) used L.E. plasma mixed with animal marrow (dog, rat, rabbit, guinea-pig). These methods all depend on the same phenomenon, that is the production of L.E. cells from leucocytes of varying sources by the action of the plasma of patients suffering from acute disseminated lupus erythematosus Berman *et al.* (1950) have shown that marrows of patients with miscellaneous disorders may vary markedly in their ability to produce L.E. cells when mixed with a potent plasma. This suggests that these auxiliary methods have no advantages over the simpler methods of examining the marrow and peripheral blood of the patient himself, and in fact may be inferior to them.

Specificity of the L.E. Cell Phenomenon.—Lupus erythematosus cells are found very frequently in acute disseminated lupus erythematosus (Montgomery and McCreight, 1949 ; Haserick, 1950 ; Hargraves, Richmond, and Morton, 1948 ; Hargraves, 1949 ; and Berman *et al.*, 1950). The cells are found more frequently with increasing activity of the disease and are less frequent or even absent in spontaneous or induced remissions. They have been seen in isolated cases of multiple myelomatosis (Hargraves, quoted by Berman *et al.*, 1950), in leukaemia (Vonder Heide, quoted by Berman *et al.*, 1950) in pernicious anaemia in relapse, in dermatitis herpetiformis, in chronic discoid lupus, in chronic and subacute disseminated lupus erythematosus, and in " probable collagen disease, undetermined type " (Berman *et al.*, 1950). Nevertheless, close investigation has in general failed to reveal L.E.

cells in the so-called "collagen diseases," rheumatic fever, rheumatoid arthritis, scleroderma, dermatomyositis, periarteritis nodosa, subacute and chronic disseminated lupus erythematosus, and varying conditions like hepatic cirrhosis and unspecified leucopenias and hyperglobulinaemias (Haserick, 1950a, and Montgomery and McCreight, 1949). I have studied about 30 marrow preparations from patients suffering from a variety of diseases; in no case were L.E. cells found. However, it should be emphasized that the free unphagocytosed, purple-staining masses which are so frequent in disseminated lupus erythematosus are far less diagnostic than L.E. cells and may even be found in normal marrows.

Histogenesis.—The inclusion bodies in the L.E. cells and the purple-staining free masses are nuclear in origin. They stain with Feulgen's reagent and with methyl green and show a strong absorption at 2537 angstroms (253.7 mμ), all of which indicate that they contain desoxyribose nucleic acid (Gueft, 1950). Morphological studies confirm this. Degeneration of nuclei can be seen in suitable marrow material. The affected nuclei become smudged and homogeneous and stain a purple colour, producing the free masses. The masses are then ingested by normal-looking leucocytes, thus producing the typical L.E. cells.

Berman *et al.* (1950), Klemperer, Gueft, Lee, Leuchtenberger, and Pollister (1950) and Klemperer, Gueft, and Lee (1949) feel that the L.E. cell is the counterpart of the haematoxylin-staining bodies found in various organs in disseminated lupus erythematosus. Nevertheless, the significance of the L.E. phenomenon and its relationship to the pathogenesis of disseminated lupus erythematosus must, for the moment, remain speculative. It has not even been established that the nuclear degeneration and production of L.E. cells occur *in vivo*.

Summary

The finding is reported of lupus erythematosus cells in the bone marrow and peripheral blood of four patients with acute disseminated lupus erythematosus.

The techniques which may be used in searching for these cells are discussed. The cells cannot be found in absolutely fresh preparations. An anticoagulant must be used and a pause of at least 10 to 15 minutes is necessary; any anticoagulant (heparin, oxalate, or citrate) may be used.

The factor producing these phenomena resides in the plasma of the patients and not in their leucocytes.

Lupus erythematosus cells are very commonly found in acute disseminated lupus erythematosus, but only very exceptionally in other diseases.

My thanks are due to Dr. J. V. Dacie for his advice and encouragement, and to Mr. E. V. Willmot, F.R.P.S., for the photomicrographs.

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FACTORS AFFECTING THE SPEED OF AGGLUTINATION IN THE COOMBS TEST

BY

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The Coombs test (Coombs, Mourant, and Race, 1945*a*, 1945*b*, 1946) has become popular as a sensitive and reliable method of detecting incomplete antibodies. The essential reagent is the serum of an animal (usually a rabbit) which has been immunized with human globulin.

Potent rabbit sera sometimes show a prozone phenomenon (Wootton, 1950; Van Loghem, Kresner, Coombs, and Fulton Roberts, 1950). The experiments described here give details of this phenomenon, and provide information on the conditions necessary for maximum sensitivity in the Coombs test.

Experimental

The reactions studied have all been of the indirect type, in which human red cells were sensitized by incubation with human sera containing incomplete anti-*D* antibody. For brevity the human sera will be called "anti-*D*," and the rabbit anti-human globulin sera will be denoted "A.H.G."

Method.—Tubes containing red-cell suspension and anti-*D* were incubated at 37° C. for 30 minutes unless otherwise stated. The cells were then washed three times (by centrifuging) with large quantities of saline, followed by the addition of sufficient saline to make a 10% suspension. A standard volume of each suspension was placed on a white tile and an equal volume of A.H.G. was added and rapidly mixed. The mixed drops were spread over a known area and the tile was rocked and transilluminated.

The time from the start of rocking to the appearance of the first naked-eye agglutination was measured to the nearest second. Observation was continued for five minutes.

Red Cell Suspension.—A standard volume of a 20% washed suspension in saline was placed in each tube. The suspensions were freshly made each day, using the same donor of genotype R_1r(CDe/cde).

Anti-D.—Several sera were used. They were obtained from women who had become sensitized during pregnancy. Tests for saline agglutinins were negative.

A.H.G.—Two samples were used, prepared by the method described by Wootton (1950). Both reagents were potent, *M.C.*2 being about four to eight times as strong as *M.C.*1 The former readily agglutinated sensitized cells when it was diluted 1 in 100,000.

Results

Effect of Diluting A.H.G.—A large stock of sensitized and washed cells was prepared and agglutinations were performed with equal volumes of cell suspension and various dilutions in saline of two samples of A.H.G. The dilutions ranged

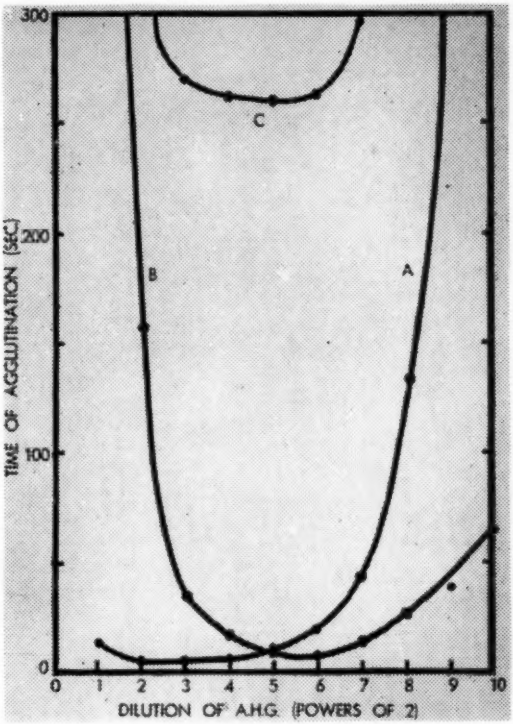


FIG. 1.—Effect of diluting A.H.G. Curve A, using A.H.G. 1; curve B, A.H.G. 2; curve C, A.H.G. 2 agglutinating weakly sensitized cells.

from 1 in 2 to 1 in 1,024. The anti-*D* used (MU) caused strong but not complete sensitization of the cells.

The results are shown in Fig. 1. For each sample of A.H.G. there was an optimum concentration at which agglutination occurred most readily. Agglutination was delayed if the reagent was used either at higher or lower dilutions.

To determine whether the same prozone occurred with weakly sensitized cells, the experiment was repeated using cells sensitized with anti-*D* diluted 1 in 16. A similar optimum concentration was found, although agglutination was, of course, delayed.

Effect of Diluting anti-*D*.—Sera containing anti-*D* were serially diluted with normal serum containing no incomplete antibody. One volume of each dilution was then used to sensitize one volume of red cell suspension. Typical results are shown in Figs. 2 and 3.

In Fig. 2 the A.H.G. was used at a dilution greater than its optimum. Agglutination therefore occurred relatively slowly. With sample MU of anti-*D*, dilution of the antibody increased the time for agglutination in a fairly regular manner. Serum EC, on the other hand, contained more anti-*D*, and dilution did not affect agglutina-

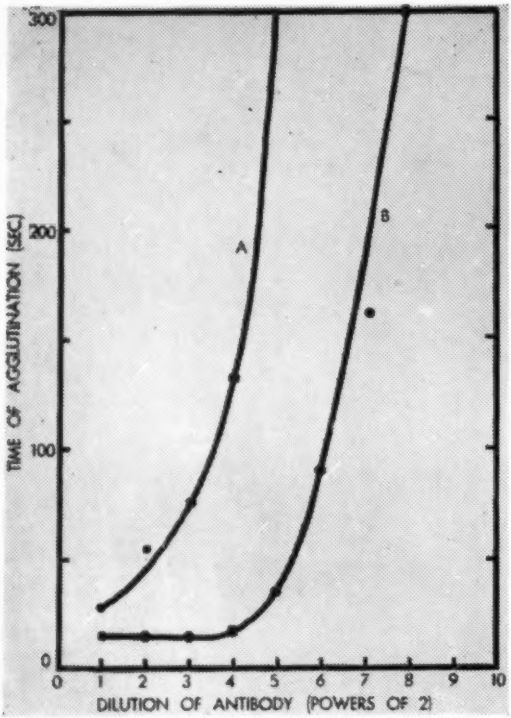


FIG. 2.—Effect of diluting the antibody, when the A.H.G. is used at a dilution greater than its optimum. Curve A, anti-D (MU); curve B, anti-D (EC).

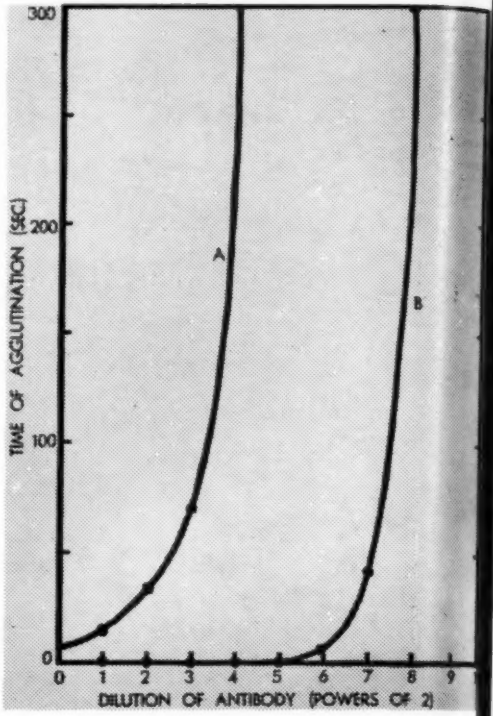
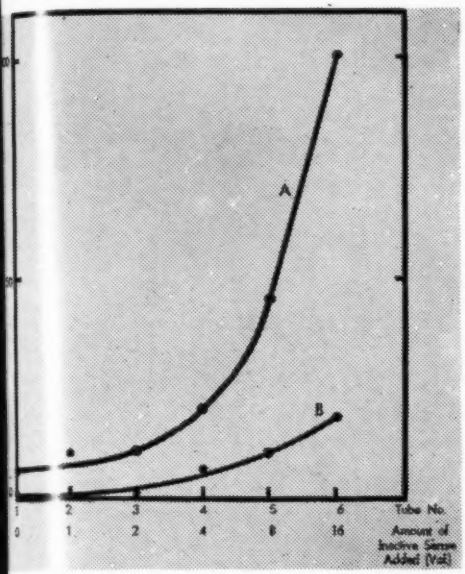


FIG. 3.—Effect of diluting the antibody, when the A.H.G. used at its optimum dilution. Curve A, anti-D (MU) curve B, anti-D (EC).

tion when the serum was present at a concentration of 1 in 16 or greater. It is concluded that this minimum time represented the time required to agglutinate fully sensitized cells.

This conclusion was supported by the results shown in Fig. 3, which refers to a similar experiment in which the A.H.G. was used at its optimum titre. With anti-*D* serum MU there was a similar relation between antibody concentration and agglutination time. As before, anti-*D* serum EC produced agglutination in the minimum time when present in concentration greater than 1 in 16 or 1 in 32. Under these conditions, A.H.G. at its optimum titre was agglutinating fully sensitized cells. The agglutination occurred instantaneously as the drop was being mixed, so that the red cell mass broke into coarse fragments as soon as the tile was tilted.

Effect of Adding Normal Serum to anti-*D*.—A series of numbered tubes was prepared, each tube containing one volume of anti-*D* serum MU. Inactive normal serum was then added to tubes 2 to 6 in the amounts specified in Fig. 4. Red cell suspension (1 volume) was then added to each tube and the contents were well



4.—Effect of adding inactive serum. Curve A, when incubated 30 min. ; curve B, incubated 4 hrs.

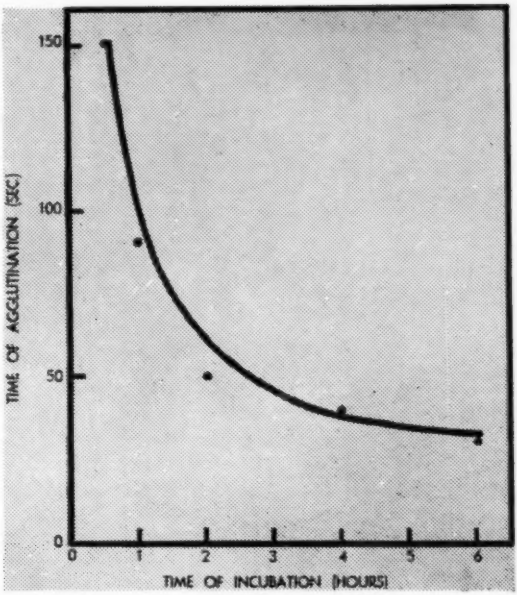


FIG. 5.—Effect of the time of incubation on the agglutination.

mixed. After incubation for 30 minutes the cells were washed and tested as usual (Fig. 4).

Each tube contained the same quantity of antibody and red cells. In spite of this, agglutination was delayed by the dilution of the anti-*D* with normal serum. Further experiments showed that sensitized cells did not lose their coating of anti-*D* when incubated with saline or normal serum. Thus the weak reactions were presumably caused by the cells' failure to combine with the available antibody when it was in dilute solution.

The experiment was then repeated, using an incubation period of four hours, during which the cell suspensions were shaken at 30-minute intervals. As shown in Fig. 4, longer incubation has diminished the dilution effect.

Effect of the Incubation Period.—A quantity of anti-*D* was suitably diluted with normal serum and mixed with an equal quantity of red cell suspension. The mixture was then incubated for six hours, being shaken each half-hour. At different times portions were removed and the red cells immediately washed. Testing followed in the usual manner.

The curve of Fig. 5 demonstrates that, under these conditions, a 30-minute incubation period is definitely not long enough to produce the strongest reaction. There appears to be little advantage from prolonging incubation beyond six hours.

This experiment was repeated with constant gentle mixing during incubation. No change could be detected in the results.

Discussion

The prozone displayed by potent preparations of A.H.G. is a potential danger, in that weak reactions may be missed if the A.H.G. used is too concentrated. Each preparation that has been tested, if potent enough, has shown an optimum dilution, which can readily be determined by a procedure similar to experiment 1.

The position is more serious if the optimum dilution is different when the A.H.G. is used to detect sensitization of cells by an incomplete antibody other than anti-*D*. This is a point about which information is required. Nevertheless, the above results show that maximum sensitivity cannot be attained with A.H.G. as made in this laboratory unless it is sufficiently potent to show a prozone.

It seems that the following conditions are required for the discovery of the weakest incomplete antibodies: (1) a large volume of serum compared with red cell suspension ; (2) incubation for a prolonged time (e.g., four hours) with frequent shaking ; (3) use of a potent A.H.G. at its optimum dilution.

In considering the problem of standardizing A.H.G., two methods present themselves. The dilution at which the A.H.G. becomes inactive may be determined ; if it is a potent reagent, this will be more than 1 in 100,000. Alternatively, the optimum dilution can be estimated. The former determination requires very large serial dilutions and is affected by the degree of sensitization of the cells, the temperature, time of observation, etc. The latter estimation is easier and does not seem to be affected much, if at all, by the degree of sensitization. Fully sensitized cells should not be used—they agglutinate too quickly—but conditions can be chosen such that the first agglutination occurs after about 10 seconds. Such an estimation will then provide a convenient way of standardization, provided further work shows that the prozone is due to a simple excess of A.H.G. in the sensitized cell *v.* A.H.G. system.

Summary

The prozone displayed by potent preparations of anti-human globulin in the Coombs test is described. Conditions necessary for maximum sensitivity in the test are given, and a suggestion is made on the subject of standardizing anti-human globulin sera.

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EFFECTS OF CORTISONE ON EXPERIMENTAL GLOMERULONEPHRITIS

BY

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Earlier attempts to produce glomerulonephritis in immune and sensitized animals with bacterial antigens gave equivocal results. The full picture of an acute glomerulonephritis (type 1 nephritis of Ellis, Evans, and Wilson, 1947) has not been achieved in such experiments. The closest approach in experimental reproduction appears to be the so-called "nephrotoxic nephritis" of Masugi (1933, 1934). This is produced by injecting a suspension of renal tissue from one species, e.g., rabbit, into another species, e.g., duck. Serum from the latter sensitized animals, anti-rabbit-kidney duck serum, when injected into the original species, rabbit, produces glomerular lesions in which epithelial and endothelial proliferations and crescent formation may be prominent, and ultimate fibrosis occur. It has been established that the active agent in the nephrotoxic serum is an organ specific antibody, as it is removed by absorption of the serum by the kidney, but not by other tissues of the species. The pathogenetic relation between nephritis produced by an antibody of the nature of nephrotoxin and the human glomerulonephritis has, however, been obscure, and Masugi himself realized that it would be more convincing to elicit glomerular lesions with an antigen rather than with an antibody. Subsequent examinations of this nature were clearly less convincing than those caused by nephrotoxic serum. The question of the pathogenesis of glomerulonephritis has been elucidated experimentally in the work of Ahlström (1936).

In the experiments reported here marked glomerular lesions were produced, corresponding to the various changes seen in the natural disease, including the type 1 and type 2 nephritis of Ellis.

Incidence of Glomerular Lesions and Albuminuria in Hyperimmunized Rabbits

In a previous paper the action of cortisone on certain morphological changes observed in rabbits immunized with a formaldehyde-killed Pfeiffer bacillus culture administered in intravenous injections three times a week for many months (Teilum, Engbaek, and Simonsen, 1950) was studied. In all the animals treated with cortisone (20 mg. hypodermically daily for up to six days), a marked regression of the massive accumulations of plasma cells in the spleen was found and further hyalinosis of

the splenic reticulum proper. Also, it was demonstrated in some cases that cortisone promoted the deposition of a homogeneous substance in the reticulum cells of the perifollicular zone of the spleen.

In such hyperimmunized rabbits in addition to plasma cell accumulations morphological lesions were found in various organs, including glomerular lesions of the kidney ; allergic granulomas and necroses, most frequently in the lungs ; vascular changes similar to periarteritis nodosa ; and marked cortical hyperplasia of the adrenals.

In an unpublished series of 42 immunized rabbits, six showed no changes, 16 slight, and 13 pronounced glomerular changes in the form of deposits in the tufts of a homogeneous substance (Fig. 1), whereas in five cases there were large amounts of hyaline material accumulated in the central parts of the tufts (Fig. 2) corresponding to the highly characteristic lesion which accompanies type 2 nephritis. Lastly, two subjects (2922 and 3197) displayed the typical picture of acute and subacute type 1 nephritis, in which endothelial and epithelial proliferation and crescent formation (Fig. 3) were conspicuous.

In some cases moderate albuminuria and possibly haematuria were also found a few months after immunization. Thus eight out of 69 animals had abnormal constituents in the urine after six months' immunization, but only one out of 85 animals after two months' immunization. After a year only 27 out of 200 rabbits were alive, and five of them had very marked albuminuria but no haematuria.

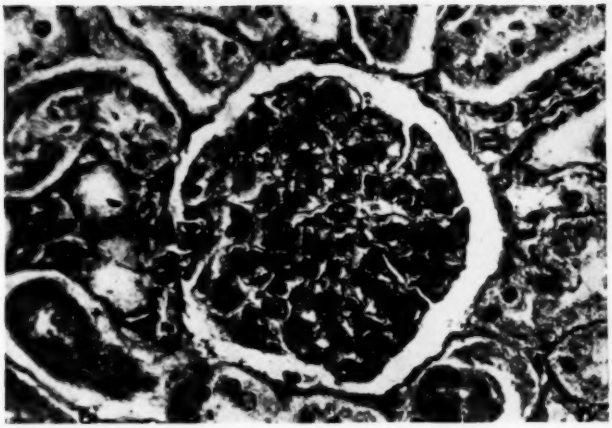
In the present paper the morphogenetic features of glomerular lesions are studied in hyperimmunized animals with pathological findings in the urine, and corresponding to the human glomerulonephritis types 1 and 2, and the pathogenesis of nephritis and the relation between the types of nephritis mentioned have been illustrated by investigations into the effects of cortisone on the glomerular lesions.

Material and Methods

All the subjects were white male rabbits of the same strain, and were immunized with a formaldehyde-killed Pfeiffer bacillus culture administered in intravenous injections three times a week (Engbaek, 1949). In eight animals immunized for seven to 16 months and showing severe albuminuria but no unquestionable haematuria, a laparotomy was performed under nembutal anaesthesia seven days after the last injection, with resection of the distal quarter of the spleen and about 1 g. of renal tissue from the left upper renal pole. The microscopical examination of the material removed by means of biopsy showed that only five out of the eight animals displayed marked glomerular changes, whereas accumulations of plasma cells in the spleen were conspicuous in all the subjects.

The five animals with glomerular lesions were given injections of cortisone in the same dose as was employed in previous experiments (Teilum *et al.*, 1950) : 20 mg. of cortisone acetate " Merck " were administered in one daily hypodermic injection. The injections were begun five days after the operation and were given in series of six days' duration interrupted by one day on which another biopsy of the kidney, and in some cases also of the spleen and the liver, was made. This treatment was continued until the animals died. In the case of one of the animals, which lived unusually long, the dose of cortisone was reduced gradually (compare the report below). The animals which did not display any glomerular changes were not treated with cortisone, but were killed at various times later on. On comparison between biopsy of the spleen

FIG. 1



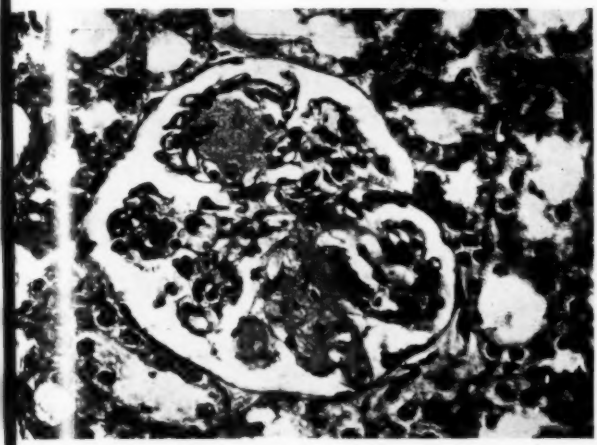


FIG. 2

FIG. 3

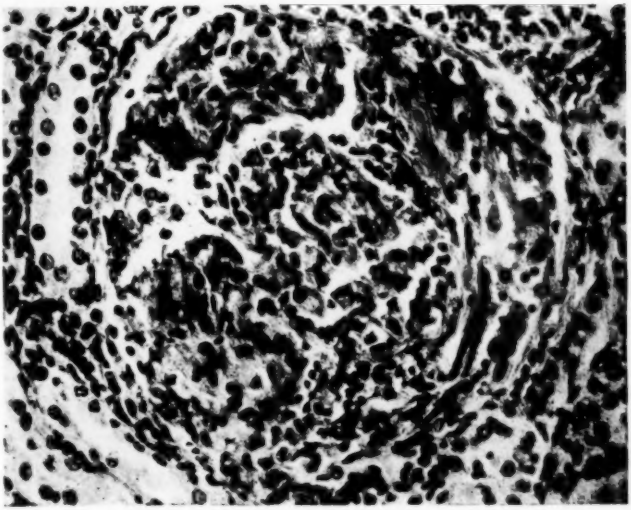


FIG. 1.—Deposits in the glomerular tufts of homogeneous prehyaline substance. Haematoxylin and eosin. × 400.

FIG. 2.—Hyaline material accumulated in the central parts of the glomerular tufts corresponding to the lesions in type 2 nephritis. Haematoxylin and eosin, × 370.

FIG. 3.—Typical picture of acute glomerulonephritis (type 1 nephritis) with crescent formation and conspicuous endothelial and epithelial proliferation. Haematoxylin and eosin, × 360.

and post-mortem microscopy of the spleen a certain indication of the spontaneous alteration of the splenic changes after discontinuance of the immunization was found.

Serum for determining the total protein (biuret reaction) was taken from all the eight animals a few days after the last immunizing injection, and electrophoretic examination of the serum was made in the five animals with glomerular lesions. Blood urea was determined in the same five animals before the first operation, and in some cases also later (Conway's micromethod). The blood pressure was determined immediately after the first injection of cortisone, and several times later on. Grant and Rothschild's capsule was used for the determination ; it was placed over the middle artery of the ear after wiping the ear with xylol. The values stated show the average of three determinations.

Results

Laboratory data stating the condition of the animals before the first operation and the beginning of the treatment with cortisone are given in Table I. The blood pressure was normal (60–80 mm. Hg) in all the subjects except one (3785). Of

TABLE I

LABORATORY AND CLINICAL FINDINGS IN ANIMALS INJECTED WITH CORTISONE

Rabbit No.	Urine	Total Protein (%)	Serum Protein Fraction / Total Protein × 100				Blood Urea (mg.%)	Blood Pressure	Survival Time after Cortisone Injection (days)
			Albumin	α-globulin	β-globulin	γ-globulin			
3252	++ albumin. A few granulated casts	6.4 6.4	50.0	8.5	18.5 (β_1) 2.5 (β_2)	20.5	33	60	21
3321	+++ albumin	3.60 3.58	26.0	16.5	37.5	20.0	38		1
3564	+++ albumin	4.15 4.09	36.5	24.5	25.5	13.5	25	70	17
3736	+++ albumin	6.89 6.83	38.5	8.5	13.5	39.5	61	70	11
3785	+++ albumin; a few granulated casts	6.09 6.07	48.0	11.0	17.0	24.0	185	105	46
3545 3599 3755	++ +++ +	5.76 6.05 7.09							No cortisone treatment
Average of 53 normal rabbits			61.0	8.3	13.8	16.9			
Average of 9 hyperimmunized rabbits previously reported (Teilum *et al.*, 1950)			56.5	4.4	14.9	24.3			

the blood urea values one was unquestionably elevated (the same subject), and another presumably slightly elevated (3736).

Case Reports

(1) Acute Glomerulonephritis (Type 1 Nephritis) in Rabbit 3785.—On October 8 blood urea was 185 mg.% ; on October 13, biopsy of spleen and left kidney was performed. On October 17 blood pressure was 105 mm. Hg, and between October 17 and 23, 20 mg. of cortisone was injected daily. On October 24 biopsy of the left kidney and spleen was performed. The abdomen contained more fluid than at the first operation. From October 25 to 30, 20 mg. of cortisone was given daily. On October 29 blood pressure was 100. On October 31 biopsy of the right lower renal pole was performed ; blood pressure was 100 mm. Between November 1 and 3, 20 mg. of cortisone was given daily, and between November 4 and 6, 10 mg. of cortisone daily. From November 7 to 20, 5 mg. of cortisone was given daily. On November 9 blood pressure was 80 mm. and blood urea 36 mg.% ; on November 11 blood urea was 32 mg.%, and on November 20, 58 mg.%. On November 21 a biopsy was taken of the convexity of the left kidney, of the spleen, and of the right lobe of the liver. On November 27 the blood pressure was 45 mm. ; urine+albumin, but microscopically nothing abnormal was seen ; Hb (% Sicca) 55. From November 27 to December 1 20 mg. of cortisone was injected daily. Death occurred on December 2.

At necropsy no oedema or exudates were seen ; the spleen was embedded in adhesions ; the liver was very pale, the kidneys enlarged and paler than normally. The loss of weight from the time of the first operation was 12%. The adrenals weighed 166 mg. per kg. of body weight.

Microscopical Observations.—The first biopsy was performed on October 12, 1950, before treatment with cortisone.

The kidney displayed the characteristic picture of acute diffuse glomerulonephritis. The glomeruli were large, prominent, and richly cellular (Fig. 4). Not only proliferation of endothelial and epithelial cells of the tufts was evident, but also proliferation of extracapsular mesenchymal cells, in particular round the afferent arteriole (Fig. 5). The capsular space contained precipitates and scattered synechiae. There were only focal periglomerular and intertubular hyaline changes. Using Unna-Pappenheim's methyl-green-pyronine stain particularly instructive pictures were obtained, which at this stage showed a very pronounced pyroninophilia of the proliferating endothelial and epithelial cells in the glomeruli, the periglomerular and scattered smaller intertubular and perivascular cell proliferations, so that with this stain the glomeruli appeared bright red in contrast to the remaining portion of the renal parenchyma. The epithelium of the parietal capsular layer was also stained in parts with pyronine, and the interstitial renal tissue displayed scattered accumulations of typical plasma cells. In some areas transition from pyroninophilic glomerular cells varying in shape and size to a homogeneous, non-pyroninophilic substance was observed.

The spleen displayed numerous massive accumulations of plasma cells, both around the follicles and in the pulp.

The second biopsy was performed on October 24, 1950, after treatment with cortisone.

The histological picture of the kidney showed striking changes in comparison with biopsy I. The glomeruli were not rich in cells now, but in the tufts there were deposits (Fig. 6) of a homogeneous non-eosinophilic substance which stained a bluish-grey with haematoxylin and eosin and a pale brown with van Gieson-Hansen, and formed larger masses in some of the glomeruli. The latter were completely cleared of cellular proliferation. Unna-Pappenheim staining showed at the same time a pronounced reduction of pyroninophilic substance in the glomeruli and extracapsular accumulations of cells ; the

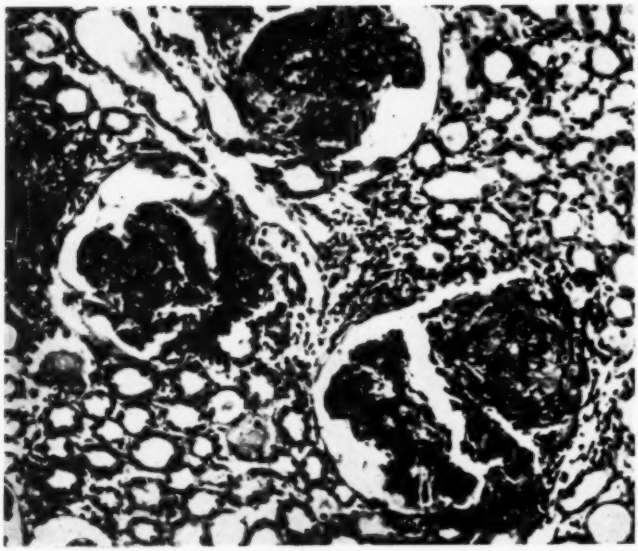


FIG. 4.—Rabbit 3785 with type I nephritis before treatment with cortisone. Haematoxylin and eosin, × 180.

FIG. 5.—Rabbit 3785 before treatment with cortisone. Proliferation of glomerular and extracapsular mesenchymal cells. Haematoxylin and eosin, × 370.

FIG. 4

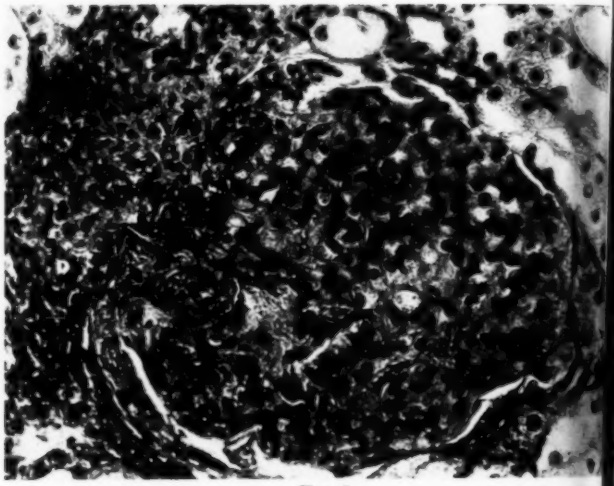


FIG. 5

glomeruli were also much poorer in cells and less conspicuous than before the treatment with cortisone (Fig. 7).

Both the spleen and the kidney displayed a marked regression of the accumulations of plasma cells.

Material was obtained by a third biopsy on October 31, 1950.

In the kidney the homogeneous substance was more compact and more deeply stained with van Gieson staining. Corresponding to the accumulations of pyroninophilic cells found in biopsy I around the vas afferens, there were now in many parts hyaline deposits in the form of local fibrosis, which also appeared around the glomeruli, perivascularly and interstitially, in particular in the medulla corresponding to the localization of

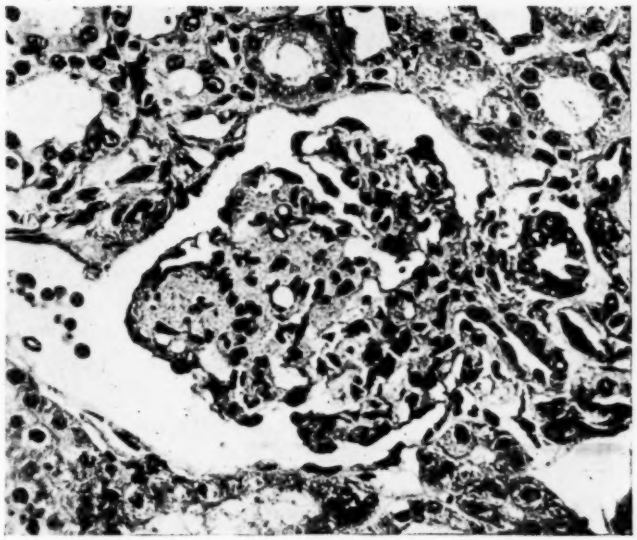
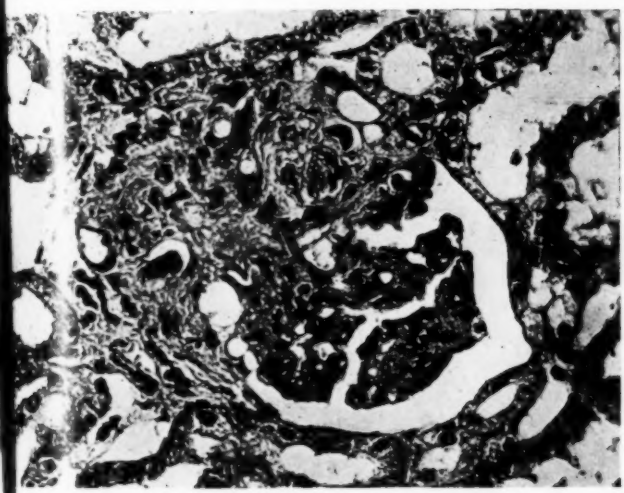


FIG. 6.—Rabbit 3785. Biopsy II after treatment with cortisone. The cellular proliferation (cf. Figs. 4 and 5) has been replaced by homogeneous deposits similar to the picture of type 2 nephritis. Haematoxylin and eosin, × 370.

FIG. 7.—Rabbit 3785. Biopsy II after treatment with cortisone showing pronounced hyaline changes corresponding to the extracapsular cell proliferation. Haematoxylin and eosin, × 370.



pyroninophilic mesenchymal cells in biopsy I. The pyroninophilia of the glomerular cells had subsided, and only few scattered plasma cells were now found interstitially.

Biopsy IV was performed on November 21, 1950.

The kidney displayed a denser homogeneous substance in the tufts of the glomeruli poor in cells, and stained a deep blue with haematoxylin and eosin. Otherwise there was no essential change from biopsy III. The spleen showed marked perifollicular and perivascular hyalinosis and regression of accumulations of plasma cells. The hepatic cells displayed marked glycogen storage with distended pale cells. In addition there were numerous small sudanophilic vacuoles.

The necropsy was performed on December 2, 1950.

With van Gieson stain the kidney showed hyalinization in many parts in, and especially around, the glomeruli. The histological picture resembled a so-called type 2 nephritis. No crescents or synechiae were seen. The glomeruli were poor in cells. The spleen showed the same conditions as in biopsy IV. The liver displayed a variegated picture, with a mixture of glycogen-filled and normal cells with granular cytoplasm. The adrenals were moderately hypertrophic

(2) Subchronic Glomerulonephritis (Type 1 Nephritis) in Rabbit 3736.—On October 13 biopsy from the left upper renal pole and distal part of the spleen was performed. On October 17 the blood pressure was 70 mm. Hg, and from that date till October 23 20 mg. of cortisone was injected daily. On October 23 the blood pressure was 70 mm. On October 24 biopsy from the left lower renal pole and proximal part of the spleen was performed. The abdomen contained more fluid than at the first operation. From October 25 to 27, 20 mg. of cortisone was given daily. Death occurred on October 28.

At necropsy no oedema or effusions were seen. The loss of weight was 14% from the first operation. The weight of the suprarenal body was 312 mg. per kg. of body weight.

Microscopical Observations.—Biopsy I was performed on October 13, 1950, before treatment with cortisone.

As in rabbit 3785, the kidney showed a typical so-called type 1 glomerulonephritis, but at a subchronic stage with pronounced epithelial crescents and capsular adhesions (Figs. 8 and 9). The glomeruli were rich in cells, but not so large as in the acute stage. A few of the glomeruli displayed slight hyaline changes, and in most parts hyalinization was seen around the glomeruli and between the tubules with signs of incipient contraction. There were numerous pyroninophilic cells in the glomeruli in the interstices and perivascularly. The spleen displayed accumulations of plasma cells, but no hyalinosis.

Biopsy II was performed on October 24, 1950, after treatment with cortisone.

In the kidney the number of cells was not increased now, and the pyroninophilia of the glomeruli and the crescents had subsided (Fig. 10). A distinct hyalinizing effect was observed on the glomerular tufts, which, with van Gieson staining, displayed fine hyaline deposits and a few hyaline globules.

Necropsy was performed on October 28, 1950.

In the kidney increasing hyalinization of the glomerular tufts and decrease of cellular proliferation was seen, and also hyalinization of extracapsular cellular accumulations. Some capsular adhesions were visible. As in rabbit 3785, the histological picture after the administration of cortisone corresponded to a nephritis of type 2, and only the capsular adhesions were reminiscent of the original picture. The pyroninophilia had subsided. A few cells of the parietal capsular layer displayed hyalinization. In the spleen there was severe fibrosis around follicles and vessels. The liver showed a variegated picture ; the cells were chiefly without any glycogen storage. The adrenals were more hypertrophic than in the other subjects treated with cortisone, with large lipidic vacuolated cells in the intermediary cortical zone, whereas the cells in the outer part of the fasciculate zone contained no lipid. There was pronounced, diffuse eosinophilic hyperplasia of the anterior lobe of the pituitary, in parts connected with degenerative changes.

(3) Excessive Glomerular Changes comparable to Type 2 Nephritis or Amyloid Nephritis in Rabbit 3564.—On October 13 a biopsy from the left kidney and from the spleen was performed. On October 17 the blood pressure was 70 mm. Hg, and from that date until October 23, 20 mg. of cortisone was injected daily. On October 23 the blood pressure was 75 mm., and on October 24 a biopsy from the left kidney and the

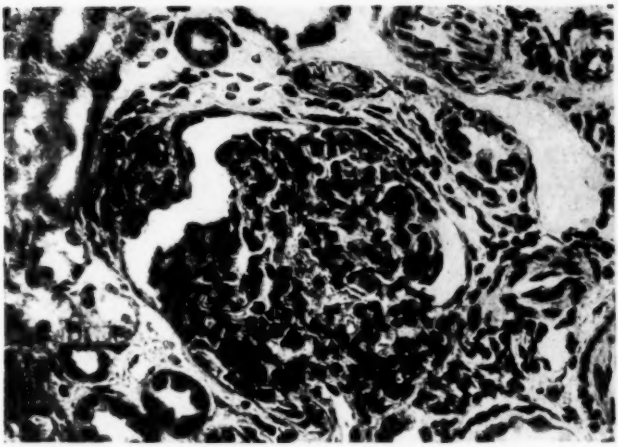


FIG. 8

FIG. 9

FIG. 8.—Rabbit 3736. Biopsy I before treatment with cortisone. Subchronic glomerulonephritis (type 1). Cellular glomerulus with crescent formation of parietal capsular layer. Van Giescn, × 370.

FIG. 9.—Rabbit 3736. Biopsy I. Glomerulus showing epithelial proliferation of parietal capsular layer. Van Gieson, × 370.

FIG. 10.—Rabbit 3736. Biopsy II after administration of cortisone. Cellular proliferation in glomeruli and synechiae replaced by homogeneous deposits. Van Gieson, × 200.

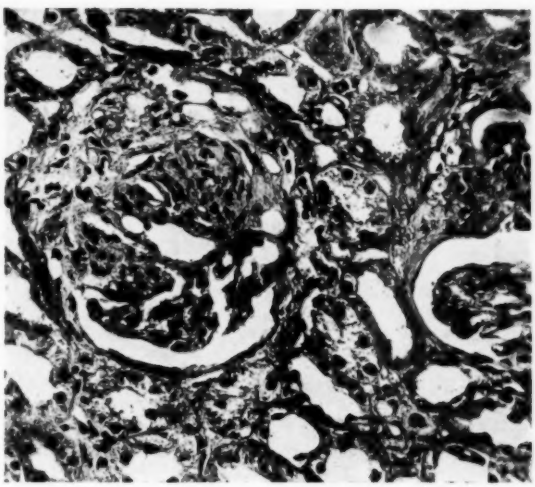


FIG. 10

spleen was taken ; slight ascites was observed. From October 25 to 30, 20 mg. of cortisone was given daily. On October 29 the blood pressure was 80 mm., and on October 31, 60 mm. On October 31 biopsy was taken from the right lower renal pole. Severe ascites and pronounced subserous oedema of the intestine were seen. On November 1 and 2, 20 mg. of cortisone was given. On November 2, the urine gave + + + albumin ; microscopy showed nothing abnormal. Death occurred on November 3.

At necropsy severe ascites and bilateral pleural exudation were seen, and atelectases and emphysema in the lungs. The weight of the adrenals was 146 mg. per kg. of body weight. The latter had decreased by 10% since the first operation.

Microscopical Observations.—Biopsy I was performed on October 13, 1950.

In all parts of the kidney there were highly changed glomeruli which were extremely poor in cells, and displayed very large deposits of a homogeneous substance in massive lumps which stained a pale yellow with van Gieson stain (Fig. 11). Scattered in the periphery there were areas with few preserved cells, which in parts were pyroninophilic. The deposits resembled amyloid, but did not seem to be so dense and did not stain with Eden's methyl violet. There were no crescents or capsular adhesions. Interstitially there were scattered, purely hyaline changes, but no contraction. In the spleen there were massive accumulations of plasma cells, and a few small granulomas with pale reticulum cells, negative on amyloid staining.

Biopsy II was performed on October 24, 1950, and III on October 31.

In the kidney the glomeruli had increased in size (cómpare Figs. 12 and 11), as the homogeneous deposits displayed a further increase, and at the same time the remaining (pyroninophiiic) cells of biopsy I had subsided. With van Gieson staining the deposits were pale yellow, and did not stain with methods for amyloid. Dilatation of the tubules was seen in parts. No fibrosis corresponding to the glomeruli but scattered fibrosis in the interstitial tissue was seen, especially around the capillaries.

Necropsy was performed on November 3, when the homogeneous deposits in the glomeruli of the kidney were now seen to be denser and completely resembling amyloid (Fig. 13), staining blue with haematoxylin-eosin stain. Eden's methyl violet stain was faintly positive in parts. Plasma cells in the spleen were decreased in number, with no amyloid deposits. There was no glycogen storage in the liver. A number of lipidic vacuoles was seen. In the adrenals there was lipid vacuolization of the intermediary zone in the cortex as in the preceding case. No unquestionable changes were seen in the pituitary, and no diffuse eosinophilia as in rabbit 3736.

(4) Rabbit 3321.—This animal, which died the day after the institution of treatment with cortisone, displayed widespread subcutaneous oedema and severe ascites in the biopsy. The renal changes were of the same nature as in rabbit 3564, with glomeruli which were poor in cells, showing large homogeneous deposits of a pale yellow in van Gieson staining ; Eden's methyl violet staining was negative. No alteration in the renal lesions were found at necropsy. The pituitary displayed no eosinophilic hyperplasia.

(5) Rabbit 3252.—On October 13 a biopsy was taken from the left kidney and the spleen. There was marked ascites. On October 18 the blood pressure was 60 mm. Hg. From October 18 to 24, 20 mg. of cortisone was injected daily. On October 24 a second biopsy from the left kidney and the spleen was taken. Slight ascites was observed. From October 25 to 30, 20 mg. of cortisone daily was given. On October 29 the blood pressure was 55 mm., and on October 31, 35 mm. On October 31 resection of the right lower renal pole was performed. The ascites remained unchanged. From November 1 to 7, 20 mg. of cortisone was given daily. On November 2 the urine contained + + + albumin. Microscopy of the urine showed nothing abnormal. On November 8 the animal died.

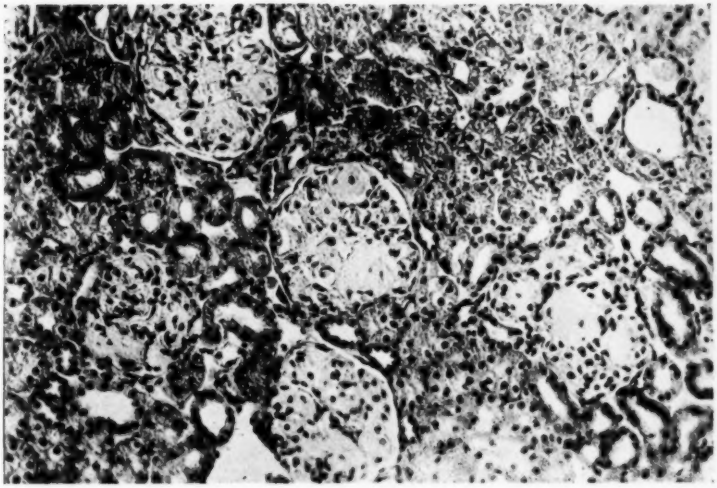


FIG. 11

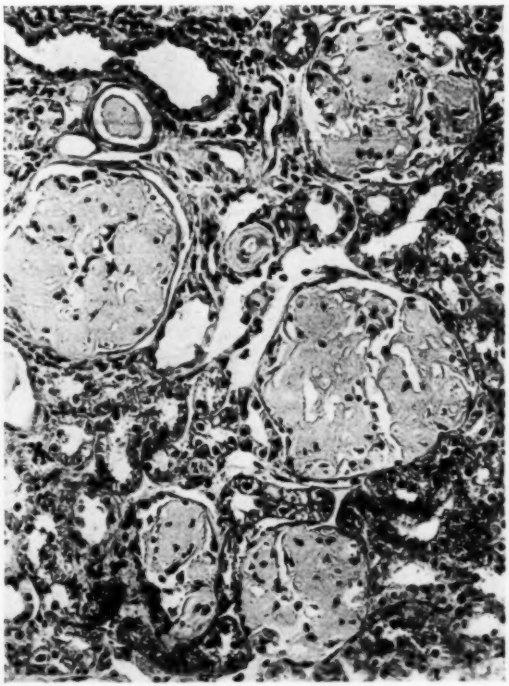


FIG. 12

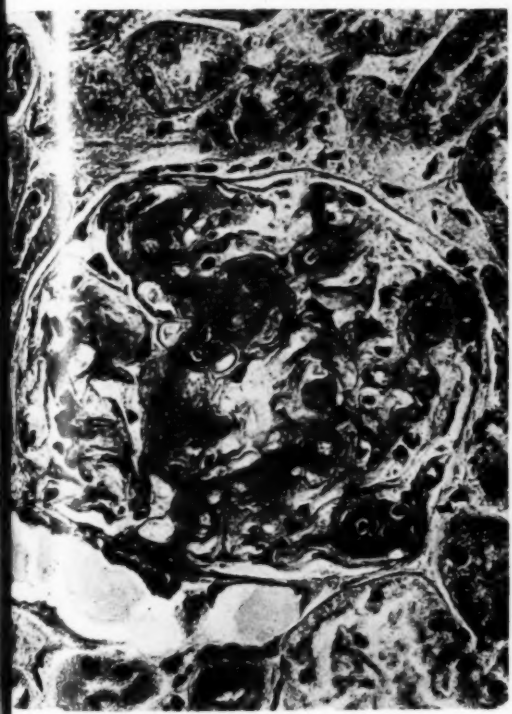


FIG. 13

11.- Rabbit 3564. Biopsy I before administration of cortisone showing ry pronounced homogeneous depo ts in the glomeruli. Van Gies , × 180.

12. Rabbit 3564. Biopsy II after dministration of cortisone. Glom ruli and deposits considerably reased. Decrease of number of c s in the glomerular tufts. Van eson, × 180.

13. Rabbit 3564 after continue dministration of cortisone. Glom uli show amyloid deposits. Haem toxylin and eosin, × 370.

Necropsy showed subcutaneous oedema, large oedematous lungs, and pleural exudation in both sides. The loss of weight was 18% since the first operation, and the weight of the adrenals 98 mg./kg. of body weight.

Microscopical Observations.—Biopsy I was performed on October 13, 1950.

The kidney displayed focal homogeneous deposits in the glomeruli of the same nature as in the preceding two rabbits (3564 and 3321). In the spleen marked pale yellow prehyaline or preamyloid deposits were seen around the follicles. Eden's methyl violet stain was negative in kidney and spleen.

Biopsy III material (October 31) and the post-mortem examination showed that the deposits in the kidney had increased, and, especially in the spleen, there were widespread amyloid-like changes, still without positive amyloid stain with Eden's methyl violet or Congo red. No hyalinization was observed of the splenic reticulum. The liver showed no glycogen storage. There was no diffuse eosinophilia of the anterior lobe of the pituitary.

The controls (3545, 3599, and 3755) operated on and not treated with cortisone were killed at the times when, respectively, 3785, 3252, and 3564 died; the former displayed none, or only slight, focal glomerular changes in the first biopsy of the kidney. These controls have been included in order to test a possible spontaneous regression of the changes of the spleen. The glomerular changes in the kidney, like the accumulations of plasma cells in the spleen, had remained unchanged at the post-mortem examination.

Discussion

The experiments reported illustrate pathogenetic and morphogenetic aspects of glomerulonephritis. In animals hyperimmunized for a prolonged period the full picture of glomerulonephritis was achieved, showing proliferations of endothelial and epithelial cells of the tufts (Figs. 3 to 5), epithelial crescents (Figs. 3, 8, and 9) and, in Case 2, also partial fibrosis. It was quite remarkable that animals treated in the same manner in some cases presented the morphological picture of acute glomerulonephritis (type 1 nephritis), whereas in others glomerulonephritis was found which was comparable to the type 2 nephritis of Ellis, showing the homogeneous prehyaline or hyaline deposits in the glomerular tufts (Fig. 2). The glomerular lesions of this type were diffuse in some of the animals, focal in others. Glomerular changes comparable to the wire loop lesion in lupus erythematosus disseminatus (Fig. 1) and the glomerular hyalinosis and paramyloidosis in sarcoidosis described by Teilum (1951) were also observed (Fig. 11).

Volhard and Fahr (1914), whose classification of renal disease has been universally employed during the last 30 years, considered glomerulonephritis to be a disease with three stages; the acute, the subacute or subchronic, and the chronic stage. During the second stage the disease may present a nephrotic syndrome. From a clinical point of view this classification has suffered from certain defects, as, clinically, typical acute glomerulonephritis generally does not tend to pass on to the nephrotic stage.

A new classification has been put forward by a group of English investigators led by Ellis (Ellis, Evans, and Wilson, 1947). According to these authors "nephritis with nephrotic syndrome" constitutes a special disease which they term glomerulonephritis type 2, as distinguished from glomerulonephritis type 1. The latter, which is a disease of children and young adults, is generally considered an

allergic reaction. The onset is abrupt, accompanied by haematuria. The oedema is not progressive, and 82% of cases recover completely. Type 2 nephritis, on the other hand, shows a much wider age distribution in man. The onset is insidious, and a history of acute infection is almost entirely lacking. Invariably progressive and persistent oedema is the presenting sign: 95% of patients die of the disease. The characteristic histological features of the two types correspond to the changes produced in rabbits.

With regard to the different symptomatology and histology of these diseases, reference may be made to Ellis's work and to A. and H. Bergstrand's survey (1949), stressing that the histology undoubtedly supports the theory that nephritis types 1 and 2 are two different diseases.

Even if glomerular lesions comparable to the two types of glomerulonephritis occurred separately in our experiments without showing signs of spontaneous transition from one type to the other, it was of special interest to observe that glomerular lesions of type 1 after the administration of cortisone acetate changed their nature completely in the direction of the changes characteristic of type 2, a fact which could be ascertained by histological examination of repeated biopsies of the kidneys from the same animals. In cases with pre- or paramyloid deposits the latter showed a further increase in the course of treatment with cortisone and, in one case, transition to amyloidosis. Morphogenetic relations between diffuse types of glomerular lesions, generally considered specific, could thus be ascertained and attributed to limited reactions from mesenchymal cells in the glomerular tufts. In this respect there was a marked parallelism with similar cellular reactions in other mesenchymal tissues under the influence of cortisone (Teilum *et al.*, 1950).

Morphogenetic Studies

The basic cellular changes were studied after treatment with Unna-Pappenheim's methyl-green-pyronine stain. In the acute type 1 nephritis the proliferating cells of the glomeruli showed a pronounced pyroninophilia, which was also demonstrated in the extracapsular accumulations of cells, in particular around the afferent arteriole (Fig. 5) and in the intertubular pericapillary tissue in the cortex and in the medulla. Sections were incubated with purified ribonuclease (10 mg. per 100 ml.) at *p*H 6.7, or with buffer solution alone for one hour at 37° C. After treatment with ribonuclease no pyroninophilia could be observed in the cytoplasm, indicating that a synthesis of ribonucleic acid was going on in the glomerular cells as well as in extracapsular mesenchymal cells in the parietal capsular layer, in the walls of the capillaries and interstitially. Many cases of the group not treated with cortisone revealed transition from large, swollen pyroninophilic cells in the tufts and the capsular epithelium to a homogeneous non-pyroninophilic substance.

It was clearly demonstrated in the cortisone experiments that this substance inhibits the pyroninophilia and promotes the transition to homogeneous precipitation (Figs. 6, 7, and 10), causing the histological picture to assume an appearance corresponding to the one found in the subjects with type 2 nephritis. Extracapsularly the number of cells also decreased, so that the accumulation of cells was replaced by a homogeneous substance (Fig. 7). The focal interstitial hyalinosis was apparently of the same pathogenesis.

The elementary effect on renal changes was thus parallel with the effect of cortisone acetate on the reticulo-endothelial tissue in the spleen previously described and with the inhibitory effect on massive (pyroninophilic) accumulations of plasma cells in the spleen of hyperimmunized rabbits (Teilum *et al.*, 1950).

A genuine hyalinization after the administration of cortisone was found in Case 2 of subchronic nephritis, in which also the proliferation of cells in the crescents decreased considerably. The findings, which we have supplemented with observations from human cases of glomerular hyalinosis, indicate that the hyalinosis of the glomerular tuft cannot be considered a thickening of the basal membrane, but must be looked upon as a product of the proliferating (pyroninophilic) mesenchymal cells. Even if the assumption of a hypersensitivity reaction is not required to explain this cellular change, which may quite naturally be considered analogous with the pyroninophilia in the spleen and in other organs, the occurrence of an increase of the plasma cells, hyperglobulinaemia, and hypersensitivity reactions is, on the other hand, a presenting finding in many conditions (Teilum, 1948 ; Robertson, 1950).

In cases showing excessive glomerular changes comparable with type 2 nephritis (Fig. 11) the treatment with cortisone also produced marked changes in the histological picture. The sparse cellular areas disappeared and further masses of homogeneous precipitates were accumulated in the tufts, causing considerable enlargement and a massive appearance of the glomeruli (Fig. 12). The histological picture suggested amyloid nephrosis, and in some areas a faintly positive reaction for amyloid was obtained in the post-mortem section after treatment by Eden's methyl violet stain for about three weeks (Fig. 13). The clinical and anatomical similarity between glomerulonephritis type 2 and amyloid nephrosis has been pointed out by A. and H. Bergstrand (1949).

In Case 1 of acute glomerulonephritis the laboratory examinations showed a fall of the blood pressure from 105 to 45 mm. Hg, and a fall of the blood urea from 185 to 36 mg.% after treatment with cortisone. The two animals with type 1 nephritis showed an increase of the content of γ-globulin in the blood before the treatment, whereas two of three animals with a marked nephrotic syndrome showed a decrease of the total protein and an increase of the α- and β-globulin fractions.

Summary

In rabbits hyperimmunized with formaldehyde-killed Pfeiffer bacillus culture given in intravenous injections for periods varying from seven to 16 months the full picture of glomerulonephritis comparable with the natural disease (Ellis's type 1 and type 2 nephritis) was achieved in some cases.

The proliferating cells of the glomeruli in acute glomerulonephritis (type 1) displayed pronounced pyroninophilia, which was abolished by treatment with ribonuclease. The morphological lesions could be attributed to definite elementary cellular reactions controlled by the hormone of the adrenal cortex.

The effect of cortisone was followed by histological examinations of repeated biopsies from the kidney, and in some cases from the spleen and the liver of the same animal. Cortisone caused a marked regression of pyroninophilic cells inside and outside the glomeruli, and promoted a transition to homogeneous precipitates in the tufts, approaching the picture characteristic of type 2 nephritis.

In cases comparable to type 2 nephritis or paramyloid syndrome, further masses of homogeneous precipitates were accumulated in the tufts, whereas the cellular areas decreased. The histological picture suggested amyloid nephrosis.

After treatment with cortisone one of the animals with acute glomerulonephritis had a marked fall in blood pressure and blood urea. The γ-globulin was increased before treatment with cortisone was instituted in the animals with type 1 nephritis, whereas cases with a marked nephrotic syndrome showed a decrease of total protein and an increase of the α- and β-globulin fractions.

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PARADOXICAL EMBOLISM

BY

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The term " paradoxical embolism " was coined by Zahn* in 1885 to describe a condition in which emboli derived from the systemic venous system reached the systemic arterial system, by virtue of an abnormal communication between the chambers of the heart.

Three such cases have been observed at necropsy at the Radcliffe Infirmary, and they are considered worthy of record in so far as they illustrate the natural history of the disease, and one of them adds to the list of those in which the diagnosis cannot be doubted.

In 1877 Cohnheim described a case which was almost certainly one of paradoxical embolism. Some writers have mentioned earlier case reports which they consider to have been examples of the condition, but it is universally agreed that Cohnheim was the first to trace the path of an embolus through a septal defect in the heart, and it seems that these earlier reports must be regarded as open to question. Even now the diagnosis must usually be a presumptive one, and it would appear difficult to make it with certainty unless the embolus could actually be found passing through the septal defect. But it was not long after Cohnheim's report that this was in fact observed. Zahn, in 1881, reported a case in which necropsy revealed thrombosis in the uterine vein, multiple systemic emboli, and a patent foramen ovale in which lay a branched thrombus. It must be admitted that there were some small thrombi adherent to the wall of the left ventricle, so that the systemic emboli may perhaps not have passed through the foramen ; but even so, this case remains an example—the first of its kind—of a paradoxical embolus " caught red-handed." In the subsequent literature there are over 80 recorded cases of paradoxical embolism. After examination of the original reports, it was felt that the diagnosis in a few of these was insufficiently established, but a further 39 case reports have been found in which the embolus lay *in situ* in the foramen ovale (Table I). Presumptive cases,

TABLE I

EMBOLISM IN SITU IN FORAMEN OVALE

X

* Thompson and Evans (1930), without giving a reference, stated that the term was suggested by von Recklinghausen, and this has been accepted by other writers. Zahn (1881) described his first case as one of " consecutive embolism," and Rostan (1884), one of Zahn's assistants, proposed the term " *l'embolie croisée* " in his doctorate thesis. Zahn (1885) rejected this and suggested " *paradox embolie* " in a paper which corrects some of the errors of Rostan's review, and in the same year von Recklinghausen (1885) put forward his theory of retrograde venous and lymphatic transport of malignant emboli; in a subsequent paper Zahn (1889) upholds his introduction of " paradoxical embolism " and discusses von Recklinghausen's paper. Welch (1899) used " aberrant embolism " for Zahn's paradoxical embolism and von Recklinghausen's retrograde embolism. Ohm (1907) correctly attributed the term " paradoxical embolism " to Zahn.

TABLE I

EMBOLISM IN SITU IN FORAMEN OVALE

Author	Age and Sex	Primary Condition	Source of Embolus	Pulmonary Condition	Sites of Paradoxical Emboli	Notes
Zahn (1881) ..	41 F	Post-partum	Iliac and uterine veins	Pulmonary emboli and infarcts	Spleen, left kidney, right iliac artery	
Rostan (1884) Case 3	46 F	Nephritis. Anasarca	Right auricular thrombus	Pulmonary infarct	None	
Case 4	58 F	Sarcoma of mesentery	Not stated	Natural	,,	
Case 5	—	Not stated	,, ,,	Not stated	Not stated	Heart only examined. A thrombus 4·3 cm. long was lying in the foramen ovale, projecting into both auricles
Hauser (1888) ..	44 M	Nephritis	Right auricular thrombus	Pulmonary emboli and infarcts	Infarcts in spleen and both kidneys	The thrombus was 5 cm. long
Bonome (1889) Case 2	40 M	Sarcoma of subscapular region	Axillary vein	Metastases	(Metastases in other organs)	
Thilo (1909) ..	—	—	Right auricular thrombus	—	—	
Versé (1909) ..	70 F	Operation for ovarian cyst	Iliac veins	Pulmonary embolism	Kidney with infarcts. Embolism of left cerebral artery	The thrombus was 13 cm. long
Mönckeberg (1909)	64 M	Pulmonary tuberculosis	Anal and prostatic veins	Pulmonary emboli and infarct	Renal infarcts	The thrombus was 6 cm. long
Schmorl (1909) ..						Two cases of "embolus in flagrante" in foramen ovale. No details
Geipel (1912) ..	48 F	Operation for uterine carcinoma	Not stated	Pulmonary embolism	None mentioned	The thrombus was 9·5 cm. long
Hannemann (1914)	54 F	Not stated	,, ,,	,, ,,	,, ,,	Very short note
Schuberth (1924) Case I	41 F	Operation for ovarian cyst	Varicose veins of leg	,, ,,	None	

TABLE I—*continued*

Author	Age and Sex	Primary Condition	Source of Embolus	Pulmonary Condition	Sites of Paradoxical Emboli	Notes
Schonig (1924) Case 2	34 F	Operation for uterine suspension	Right iliac and hypogastric veins	Pulmonary embolism	None mentioned	Thrombus was 5 cm. long
Wolff and White (1926) Case 11	43 F	Carcinoma of ovary	Pelvic veins	Not stated	Descending branch of left coronary artery	Described as "embolism of patent foramen ovale"
Wittig (1927) ..	55 F	,, ,,	Femoral and pelvic veins	Pulmonary embolism	Right subclavian artery. Innominate artery. Right common carotid artery with cerebral softening	Old bilateral renal infarcts
Thompson & Evans (1930) citing						
T. R. Elliott's case	35 F	Splenic anaemia Splenectomy	Left internal iliac vein	Pulmonary infarcts	None	The thrombus was 27·5 cm. long
F. Young's case ..	70 F	Cholelithiasis Cholecystectomy	Not stated	Pulmonary embolism	,,	The thrombus was 11 cm. long
Case 5	25 M	Teratoma testis	Malignant emboli	Natural (gross and microscopic)	Coronary arteries Right middle cerebral softening	
Barnard (1930) ..	63 M	Carcinoma of prostate	Not stated	Pulmonary embolism	Renal and splenic infarcts	The thrombus was 15 cm. long
French (1931) ..	36 M	Myomectomy	,, ,,	,, ,,	None	The thrombus was 16 cm. long
Geipel (1931) Case 1	35 F	,,	Right femoral vein	,, ,,	,,	The thrombus was 13 cm. long
Case 2	42 M	Not stated	Not stated	,, ,,	,,	The thrombus was 10 cm. long
Case 3	45 F	Ventrifixation of uterus	Right common iliac vein	,, ,,	,,	The thrombus was 8·5 cm. long
Case 4	51 F	Not stated	Right saphena magna vein	,, ,,	,,	The thrombus was 7 cm. long
Taylor (1933) ..	45 F	Bronchopneumonia	Femoral and internal iliac veins	Pulmonary emboli and infarcts	Left kidney, spleen	

TABLE I—*continued*

Author | Age and Sex | Primary Condition | Source of Embolus | Pulmonary Condition | Sites of

TABLE I—*continued*

Author	Age and Sex	Primary Condition	Source of Embolus	Pulmonary Condition	Sites of Paradoxical Emboli	Notes
Merkel (1934) Case 1	76 F	Fracture of left humerus and clavicle	Left subclavian vein	Pulmonary emboli and infarcts	None	The thrombus was 4 cm. long
Hirschboeck (1935)	74 F	Operation for strangulated hernia	Femoral veins	Pulmonary embolism	,,	
Jones (1936) ..	57 F	Operation for acute appendicitis	External iliac and femoral veins	Natural, but the day before death there was dyspnoea and cyanosis with pleural friction	Softening in left cerebral hemisphere	
Koritschoner (1936)	63 F	Operation for uterine suspension	Right iliac and femoral veins	Pulmonary embolism	None	
Ingham (1938) Case 1	45 M	Cholelithiasis Cholecystectomy	" Veins of leg "	,, ,,	Renal infarcts	The thrombus was 10 cm. long
Case 2	62 F	Cholelithiasis Cholecystectomy	Not stated	Pulmonary emboli and infarcts	Left posterior cerebellar artery	
Case 3	56 F	Operation for prepyloric ulcer	,, ,,	,, ,,	Renal infarcts	
Case 4	56 F	Operation for peptic ulcer	Iliac veins	Pulmonary embolism	None	
Case 5	48 M	Operation for carcinoma of rectum	Left femoral vein	,, ,,	,,	
Case 6	54 F	Diabetes	Not stated	,, ,,	,,	
Vimtrup (1941) Case 1	70 F	Phlebitis of varicose veins	Varicose veins of leg	,, ,,	,,	The thrombus was 10·5 cm. long
Case 2	77 F	Carcinoma of cystic duct. Secondaries in gall bladder and liver	Not stated	,, ,,	Cerebral softening	The thrombus was 10 cm. long
Young, Derbyshire, and Cramer (1948)	50 F	Uterine fibroids Hysterectomy	Inferior vena cava. Hypogastric veins	Pulmonary emboli and infarcts	Right femoral and common iliac arteries, aorta	Treated with dicoumarol; foramen ovale 9 mm. in diameter
Johnson (1951) .. Case 1	70 M	Carcinoma of prostate	Veins of right calf	,, ,,	Right femoral and left internal carotid artery	The thrombus was 7 cm. long

though doubtless more common, are less striking, but a further 43 case reports were found in which the diagnosis appeared to be virtually certain (Table II).

The case reports which have been rejected are those of Firket (1890), Buhlig (1904), Schmorl (1909), Dietrich (1925), and Armand-Delille and Lesobre (1935).

Firket's (1890) four cases showed fibrinous clots passing through a patent foramen ovale, but there were no emboli in the arterial system, no venous thromboses, and only in one case (Case I) was there a pulmonary infarct and that was associated with mitral and aortic stenosis. Buhlig's (1904) case report deals with a case of miliary tuberculosis in which the foramen ovale was patent, and he suggested that embolization of tuberculous material from a primary abdominal focus took place through the interauricular defect. Schmorl's (1909) case is of interest as he regarded it as an example of reversed embolism in which a portion of clot, formed on the walls of the left ventricle and extending down the aorta, passed through a patent foramen ovale and became lodged in the pulmonary arteries, but the account is very short.

Dietrich's (1925) case is difficult to assess. It was a patient aged 51 who had been in bed with thrombophlebitis of the leg for 11 weeks, and two days before death developed pain in the chest and hemiplegia ; at necropsy in addition to the changes in the leg vessels, the right jugular vein and transverse sinus were thrombosed, there were antemortem thrombi adherent to the wall of the right auricle, clots in the pulmonary artery, and a pencil patency of the foramen ovale ; there was a thrombosis of the right carotid artery but no evidence of embolization. Armand-Delille and Lesobre (1935) reported a case of an 18-month-old child with a complex congenital heart lesion and hemiplegia who was found to have an interventricular septal defect as well as a patent foramen ovale and large friable vegetations on the mitral valve.

In addition there are a number of German inaugural dissertations which from their title deal with paradoxical embolism, but none were available in this country and the majority were not even to be found in abstract, so they only appear in the bibliography for the sake of completeness (Ems, 1907 ; Jaenicke, 1894 ; Kunkel, 1912 ; List, 1910 ; Müller, 1925 ; Poths, 1887 ; Scheven, 1894 ; Schmieden, 1888).

Case Reports

Case 1 (A. R.) Man Aged 70 (Reg. No. 75260/47).—The patient was admitted to hospital on July 27, 1947, with symptoms of urinary obstruction of seven weeks' duration. A diagnosis was made of carcinoma of the prostate gland, with multiple secondary deposits in the bones. Treatment with stilboestrol was instituted, but on July 31 catheterization and decompression were necessary. On the night of August 2 he had a small haemoptysis, and rales were heard at the base of the right lung. On August 3, while using a bedpan, he collapsed and was found to have a right-sided hemiplegia. He died on the following day.

At necropsy (P.M. 359/47) the diagnosis of prostatic carcinoma with skeletal secondaries was confirmed. The lungs were found to be very congested, and there was a recent haemorrhagic infarct in the right lower lobe ; the right pleural cavity contained about 300 ml. of clear, straw-coloured fluid. Branches of the pulmonary artery throughout both lungs contained thrombi, and a coiled cylindrical thrombus, 5 mm. in diameter and 12 cm. in length, lay astride its bifurcation, extending into both branches, particularly into the right. There was thrombosis of the deep veins of the right calf ; distally the thrombus was adherent to the veins, but the proximal end of it (which was in the popliteal vein)

TABLE II
PRESUMPTIVE PARADOXICAL EMBOLI

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PRESUMPTIVE PARADOXICAL EMBOLI

Author	Age and Sex	Primary Condition	Source of Emboli	Pulmonary Condition	Size of Foramen Ovale	Paradoxical Emboli	Notes
Cohnheim (1877)	35 F	Not stated	Leg veins	Not mentioned	3 fingers	Embolism of middle cerebral artery	
Litten (1880) ..	43 F	Pulmonary tuberculosis	Thrombus of right auricle, leg veins	Pulmonary emboli and infarcts	Not stated	Right femoral artery. Old infarcts, kidney, spleen	Demonstrated by Virchow
Riegel (1883) Case 1	45 F	Congestive cardiac failure	Right auricular thrombus	Chronic emphysema	" Pencil "	Right radial artery. Renal and splenic infarcts	
Rostan (1884) Case 6	? F	Puerperal sepsis	Thrombosis right pelvic and saphenous veins	Pneumonia	1 finger	Infarcts of spleen and both kidneys	
Case 7 ..	65 M	Aortic regurgitation	Right auricular thrombus	Passive venous congestion	" Wide open "	Cerebral softening	
Case 8 ..	66 F	Spinal caries	Inferior vena cava	Pneumonia Pulmonary embolism	" Open "	Renal and splenic infarcts	
Case 9 ..	54 F	Congestive cardiac failure	Right auricular thrombus	Chronic emphysema Pulmonary embolism	" Wide open "	Renal and splenic emboli and infarcts Embolism right middle cerebral artery	
Case 10 ..	42 F	Pulmonary tuberculosis	Right auricular thrombus	Pulmonary tuberculosis	" Open "	Infarct right kidney	
Case 11 ..	28 M	Typhoid fever	Thrombosis of femoral veins	Pulmonary emboli and infarcts	" Wide open "	Splenic infarct	
Case 12 ..	63 M	Multiple injuries	Thrombosis right saphenous vein	Pulmonary embolism	" Wide open "	Splenic infarcts Cerebral softening	
Schmorl (1888)	? M	Railway accident Crushing injury	Rupture of liver	Fat and liver emboli	1 finger	Left auricle Left renal artery	
Marchand (1894)	69 M	Secondary syphilis	Right femoral vein	Pulmonary emboli and infarcts	1 finger	Left coronary artery. Infarcts spleen and right kidney. Softening left cerebellum	

TABLE II—*continued*

Author	Age and Sex	Primary Condition	Source of Emboli	Pulmonary Condition	Size of Foramen Ovale	Paradoxical Emboli	Notes
Rabé (1899) ..	57 M	Chronic emphysema Bronchopneumonia	Right auricular thrombus	Pulmonary infarct	2·5 cm. long	Left kidney splenic infarcts	
Hocheisen (1904)	61 F	Pneumonia	Varicose veins of leg	Pneumonia Pulmonary embolism	"Wide open"	Right vessel of Sylvian fossa Splenic infarcts	
Stoerk (1907) ..	?	Operation for umbilical hernia	Pelvic veins	Pulmonary emboli and infarcts	"Wide open"	Right kidney and spleen	
Ohm (1907) ..	16 M	Congestive cardiac failure	Haemorrhoidal veins	Natural	"Probe"	Right middle cerebral artery with softening	
Kyber (1909) Case 1	53 F	Emphysema Pneumonia	Leg veins	Emphysema Pneumonia Pulmonary embolism	"Pencil"	Arch of aorta. Innominate Right subclavian and common carotid arteries Splenic infarct 17 cm. of thrombus in aorta	Verse's (1909) Case II
Abrikossoff (1913)	Stillbirth M	Feeble infant after difficult labour	Cerebral birth injury	Not expanded	"Open"	Embolus of cerebellar tissue in left coronary artery	
Paus (1915) ..	56 M	Fracture of tibia	Veins of right leg	Pulmonary embolism	"Open"	Kidney right middle cerebral artery with softening	
Specht (1917) ..	? M	Gunshot wound Amputation left leg	Left femoral vein	Bronchopneumonia	"Wide open"	Shrapnel splinter size of nut in left ventricle	
Widerøe (1919)	54 F	Carcinoma of stomach	Not stated	Not stated	"Wide open"	Left femoral artery embolus	Died from carcinoma of stomach Treated by embolectomy

TABLE II—*continued*

TABLE II—*continued*

Author	Age and Sex	Primary Condition	Source of Emboli	Condition	Foramen Ovale	Paradoxical Emboli	Notes
Nyström (1925)	56 F	Not stated	External iliac vein	Pulmonary embolism	" Wide open "	Cerebral emboli Emboli in common iliac arteries	Treated by embolectomy but died of cerebral symptoms
Hensel (1921) ..	41 F	Gravid fibromatous uterus Hysterectomy	Pelvic veins	Bronchopneumonia with gangrenous area	1 finger	Right middle cerebral artery with softening Superior mesenteric artery	
Beattie (1925) Case 1	30 F	Chronic appendicitis Appendicectomy	Leg veins	Pulmonary embolism	0·6 cm.	Right common iliac artery Renal infarct	
Case 2 ..	49 M	Congestive cardiac failure	Thrombus in right auricle	Passive venous congestion. No infarcts or emboli	3+2 cm. small interventricular defect	Recent and old renal infarcts	
Winkelbauer and Urban (1929)	33 M	Post-appendicectomy	Left femoral vein	Pulmonary embolism	" Pencil "	Left middle cerebral artery	
Dahl-Iversen (1930)	53 F	Varicose veins	Right saphenous vein	Not stated	Patient survived	Left femoral artery	Embolectomy Recovered
Thompson & Evans (1930) Case 1	38 F	Post-appendicectomy	Right femoral vein	Pulmonary infarct	Systolic murmur over sternum	Cerebral embolism	Survived
Case 2 ..	61 M	Operation for loose body in knee	Bilateral femoral veins	Pulmonary embolism and infarcts	0·8 cm.	Cerebral embolism and softening Renal infarct	
Case 3 ..	34 M	Post-pneumonic empyema. Rib resection	Bilateral femoral veins	Pneumonia; pulmonary collapse	0·6 cm.	Splenic artery and splenic infarct Left middle cerebral artery	
Case 4 ..	30 F	Post-partum	Femoral and pelvic veins	No infarct or embolus. Pulmonary tuberculosis	0·3 cm.	Cerebral softening. Renal infarcts	
Hartfall (1931)..	49 M	Compound fracture of tibia and fibula. Amputation	Veins of leg	Pulmonary embolism	1×0·5 cm.	Basilar artery	

TABLE II—*continued*

Author	Age and Sex	Primary Condition	Source of Emboli	Pulmonary Condition	Size of Foramen Ovale	Paradoxical Emboli	Notes
Saphir (1933) Case 1	35 M	Bronchopneumonia	Right femoral vein Right auricle	Bronchopneumonia Pulmonary embolism	" Patent "	Left descending coronary and femoral arteries	
Reah (1933) ..	51 M	Hemiplegia	Left iliac vein	Pulmonary infarct	" Pencil "	Infarction of brain Thrombosis of right middle cerebral artery	
Jacobi, Kenler, and Silverman (1934)	47 F	Gastro-enteritis	Left femoral vein	Natural	0.5 cm.	Left anterior descending coronary artery	
Merkel (1934) ..	58 M	Fracture of ribs	Left subclavian vein	Pulmonary embolism	" Finger "	Common carotid artery with left cerebral softening. Left subclavian artery. Infarcts in kidney and spleen	
Neely (1936) ..	59 F	Phlebitis of right saphenous vein	Saphenous vein	Pulmonary emboli and infarcts	1·2 cm.	Superior mesenteric artery. Splenic artery. Infarction of spleen	
Ingham (1938) Case 7	39 F	Congenital heart disease	Not stated	Pulmonary thrombosis	2·5 cm.	Cerebral abscess	
Vimtrup (1941) Case 1	71 F	Senile mental change	Left external iliac vein. Right saphenous vein	Pulmonary embolism	1·5 cm.	Infarct kidney and spleen. Cerebral softening	
Case 2 ..	68 F	Cardiac failure	Thrombosis of right auricle	Passive venous congestion	6 mm.	Coronary artery Splenic artery	
Hanna (1941) ..	70 F	Fracture of right tibia	Right popliteal vein	Pulmonary embolism	1·5 cm.	Left internal carotid and middle cerebral arteries. Left subclavian artery. Superior mesenteric artery	

TABLE II—*continued*

Author	Age and Sex	Primary Condition	Source of Emboli	Pulmonary Condition	Size of Foramen Ovale	Paradoxical Emboli	Notes
Porter (1941) ..	26 M	Phlebitis of leg	Leg veins	Natural	No clinical signs	Hemiplegia	Survived
Birch (1945) ..	11 M	Congenital heart disease. Infective endocarditis	Vegetation of pulmonary valve	Not stated	Interventricular septal defect	Infarct kidney and spleen. Embolus at aortic bifurcation and left common iliac artery	
Beaver and Ellis (1947)	40 F	Anterior and posterior colporrhaphy	Thrombus in Chiari's network	Bronchopneumonia Pulmonary embolism	1·5×1 cm.	Splenic, renal and left tibial arteries, cerebral softening	
Johnson (1951).. Case 2	63 M	Prostatectomy	Right femoral vein	Bronchopneumonia Pulmonary embolism	Finger	Right internal carotid artery	
Case 3 ..	69 F	"Stroke"	Right femoral vein	Pulmonary emboli and infarcts	1 cm.	Renal infarcts. Embolus of aorta. Internal iliac arteries and right femoral artery. Cerebral softening	

Note.—According to Thompson and Evans (1930) the diameter of a probe can be taken as 0.2 cm. and of a pencil as 0.7 cm.

FIG. 1.—Right heart showing an embolus lying in the foramen ovale. The femoral artery containing an embolus can be seen in the upper right-hand corner of the photograph.

was lying free in the lumen, and showed a rough, blunt surface suggesting recent fracture. The heart showed no mural thrombosis. There was a valvular patency of the foramen ovale 5 mm. in diameter, through which passed a cylindrical thrombus 7 cm. in length: the main part of this thrombus lay in the right atrium, but one end presented as a coiled knot in the left atrium (Fig. 1). The right femoral artery contained a cylindrical embolus 5 cm. long, just below the inguinal ligament, with secondary thrombosis distal to it; the embolus was becoming adherent to the arterial wall. The left internal carotid artery was occluded at its bifurcation by a small embolus, distal to which the middle and anterior cerebral arteries were filled with clot.

Case 2 (E. N.) Man Aged 63 (Reg. No. 65755/47).—The patient stated that in 1920, when he was 36 years of age, he had had a right-sided paralysis which had incapacitated him for about three months, but which had subsequently cleared up completely. He was admitted to hospital on August 6, 1949, on account of difficulty of micturition; cystoscopy was performed on August 8, and benign prostatic hyperplasia was diagnosed. His blood pressure was recorded on admission as 120/95. On August 10 he had a sudden onset of right-sided weakness while at stool; he was found to have exaggerated reflexes on the right side, and an extensor right plantar response. Next day he was much better; his blood pressure was now recorded as 170/110, and the incident was regarded as having been due to hypertensive encephalopathy. On August 15 a one-stage prostatectomy (Freyer) was carried out. After this the patient's condition remained satisfactory until September 8, when he collapsed again, with a left-sided flaccid paralysis, and clonic movements in the right arm. His condition deteriorated rapidly, and he died four days later.

At necropsy (P.M. No. 421/47) there was an antemortem thrombus in the right internal carotid artery, extending to the bifurcation, with corresponding cerebral softening. Both kidneys contained recent infarcts. Fragmented antemortem thrombi were found in the right femoral vein, the heart contained no mural thrombi, but there was a valvular patency of the foramen ovale, which admitted the forefinger. The lungs were congested and oedematous, and there was a considerable degree of bronchopneumonic consolidation; many of the smaller branches of the pulmonary artery in both lungs contained antemortem thrombi, but there were no corresponding infarcts.

This case appears to be of particular interest in that it is possible to explain the observed rise in blood pressure and the subsequent cerebral thrombosis as both being due to para-

doxical embolism, the former to the renal infarcts. The nature of the neurological lesions causing the incidents of 1920 and of August 10, 1947, however, must remain very doubtful. It will be noted that Case 3 also contains the story of a transient " stroke " which likewise remains unexplained.

Case 3 (A. C.) Woman Aged 69 (Reg. No. 85923/48).—This patient was admitted to hospital on January 9, 1949, following an attack of acute dyspnoea associated with the signs of occlusion of the right femoral artery. She had had four operations for genital prolapse and stress incontinence, the last being a " urethral sling " operation in October, 1948. In December she had had a mild " stroke," affecting the right side of the body and her speech. On January 2, 1949, she had had acute pain under the right breast (severe enough to require morphine), which had lasted on and off ever since. She stated that she had always been " chesty." On January 9 there was a sudden onset of dyspnoea and inability to move the right leg, and on admission on the same day she was found to have weakness of the left arm and signs in the right leg suggestive of a femoral embolus, but no abnormality was found in the respiratory or cardiovascular systems. She was treated with heparin, but bronchopneumonia supervened and she died on January 16.

At necropsy (P.M. 35/49) the left pulmonary artery was seen to be filled by a recent embolus ; the branch to the right lower lobe was occluded by a thrombus which was just starting to organize. The inferior vena cava and the right femoral vein and its branches contained antemortem thrombi. There was a valvular patency of the foramen ovale about a centimetre in diameter. There was an embolus just above the bifurcation of the aorta, with clot extending into both external iliac arteries and into the femoral artery on the right side ; there were infarcts of both kidneys which were starting to organize, and, in the brain, numerous areas of punctate haemorrhage, with early cerebral softening. The appearance of these areas in the brain was considered to be compatible with the effects of multiple cerebral emboli ; the largest of them measured $3 \times 2 \times 1$ cm. and was situated at the upper part of the right post-Rolandic region.

Discussion

It is felt that these three cases fulfil the criteria for a diagnosis of paradoxical embolism. They all had patent foramina ovale and pulmonary emboli probably derived from phlebothrombosis of the leg veins ; they all showed systemic arterial emboli without evidence of thrombosis in the left heart, and in the first case an embolus was seen lying in the foramen ovale at post-mortem examination.

In nearly all the convincing cases of paradoxical embolism, the cardiac lesion is a patent foramen ovale. For an embolus to pass through such an orifice, four conditions must be fulfilled. First, there must be a suitable source of embolic material in the venous circulation of the right heart. Secondly, there must be a patency of the foramen ovale of suitable size, and this is by no means uncommon. Patten (1938) found some degree of patency in 20% of 4,000 unselected necropsies. Thompson and Evans (1930) in an unselected series of 1,000 cases found 35% " probe patent " and 6% " pencil patent," but of these latter, over half the cases were under 6 months of age. Thus it would appear that at least 6% of the human race have an interatrial septal defect capable of transmitting a sizeable embolus. The fact that the majority of these foramina are valvular is not particularly important, in view of the third condition ; it is only necessary that they should be capable of transmitting an embolus from right to left.

" Two-way " patency is a relatively uncommon condition. Roesler (1934) gave a good review of 64 cases, in which he showed that although a number of the affected

individuals have lived to a ripe old age, apparently unencumbered by a continual leak of blood from the right to the left heart, yet the average age at death was only 36 years. Three-quarters of them suffered from valvular disease of some sort, mitral disease being the commonest (the Lutenbacher syndrome first described by Maude Abbott in 1915). Further, the overloaded right ventricle was not normally capable of holding out indefinitely ; its ultimate failure gave rise to cyanosis—the *cyanose tardive* of Bard and Curtillet (1889)—and death. Unfortunately it is not always possible to gather from the recorded cases of paradoxical embolism which of the foramina were valvular and which were " two-way," but if, as is held by Beattie (1925), it is a fact that paradoxical embolism is more commonly found in association with " two-way " patency than with the valvular variety, then it is suggested that this is because such patencies tend to be larger and are therefore capable of transmitting a greater stream of blood, and because they are harder to overlook at necropsy, but not because they are " two-way."

Thirdly, for paradoxical embolism to occur through the foramen ovale, the pressure in the right atrium must be higher than that in the left. This may be caused, for instance, by any lesion capable of obstructing the pulmonary circulation, or by the *cyanose tardive* of the widely patent foramen ovale. But the most relevant and eminently likely cause in this connexion is pulmonary embolism. Zahn (1881) noted that paradoxical embolism was commonly preceded by pulmonary embolism ; and of the 41 cases in which the clot was *in situ* in the foramen ovale, at least 32 had pulmonary embolism or infarction ; of the remaining nine, one (Jones, 1936) had the symptoms of pulmonary embolism during life, but none was found at necropsy ; in Thompson and Evans's case 5 (1930) it is stated definitely that the lungs were normal macroscopically and microscopically, but, as this case was associated with malignant emboli, it is somewhat unusual ; Rostan's (1884) case 3 is also said to have had normal lungs, and Bonome's (1889) case showed pulmonary metastases ; in the other five, the condition of the lungs was not specified. In Jones's case it is tempting, though perhaps a little fanciful, to postulate that pulmonary embolism did in fact occur, giving rise to a spasm of the pulmonary arterial system, which caused the embolus to pass back into the right atrium and through the patent foramen.

In the 46 presumptive cases, pulmonary embolism or infarction was present in 28, absent in 13, and unknown in five, of which two cases survived the thromboembolic incident. Of the 13 cases in which pulmonary embolism or infarction was absent, four had pneumonia, three showed passive venous congestion, two had pulmonary tuberculosis, one showed chronic emphysema, and in one the lung was not expanded. There were two cases in which the lungs were stated to be natural ; Ohm's (1907) case had a large " two-way " patency and had recently become cyanosed (presumably *cyanose tardive*), but Jacobi, Kenler, and Silverman (1934) are quite specific in their statement that both the lungs and the pulmonary arteries were natural. Thus in almost every case in which full information is available, there was some potential cause of raised right atrial pressure, by far the commonest being pulmonary embolism.

At this point some mention should be made of the often quoted work of Haggart and Walker (1923). They showed, in cats, that sudden occlusion of the left pulmonary artery for a matter of minutes produced no significant alteration in the systemic

blood pressure but caused a rise in pulmonary arterial pressure averaging about 29% and seldom exceeding 33%. Further, that up to about 63% of the total pulmonary arterial circulation could be occluded without signs of cardiac embarrassment, but that above this level dilatation and failure were rapid. Their work is a contribution of undoubted value to the study of pulmonary embolism, but it is felt that its relevance to the relative pressures in the atria of the human subject suffering from such an embolism has from time to time been over-emphasized.

Fourthly, given an embolus, a patent foramen ovale, and a flow of blood through the latter from right to left, there is still required that imponderable element of chance by which the embolus passes through the foramen instead of up the pulmonary artery with the residuum of the normal circulation. It seems reasonable to suppose that a patent foramen ovale would be instrumental in prolonging life in cases of massive pulmonary embolism by providing a much-needed shunt, and that the magnitude of the stream through it might well be as great as that through the pulmonary artery.

Up to now the discussion has been confined to paradoxical embolism through a patent foramen ovale. An interventricular septal defect would seem to present an equally tempting avenue to an itinerant embolus ; and, indeed, if pulmonary stenosis or dextraposition of the aorta were also present, an embolus arriving at the right heart would seem to stand an excellent chance of emerging by way of the aorta. However, only one really convincing case has been found in which an embolus has been demonstrated which must be presumed to have passed through such a defect. Birch (1945) describes the case of a boy aged 11 years, in whom a large vegetation from the tricuspid valve passed through an interventricular septal defect, and had become lodged at the bifurcation of the aorta. But the rarity of the interventricular defect as the offending lesion is not difficult to explain. Patency of the foramen ovale is far more common than the various complexes involving an interventricular defect, and this disparity becomes even greater as age advances, in so far as the latter constitute a greater threat to life. The average age of the undoubted cases of paradoxical embolism through the foramen ovale is 51, an age at which interventricular septal defects must be extremely rare, compared, on Thompson and Evans's (1930) figures, to the 1% of the populace of this age with a " pencil patency " of the foramen ovale. Zahn (1885) stated that at Geneva in the years 1882–4, in a consecutive series of 711 necropsies, a patent foramen ovale was found in 139 (20%), and that in this period there were nine examples of paradoxical embolism (Rostan, 1884, Cases 3, 4, 6–12). In her analysis of 1,000 cases of congenital heart disease, Maude Abbott (1932) noted the occurrence of paradoxical embolism in 13 cases, but nine of these occurred amongst 90 cases in which patency of the foramen ovale was present. From these figures, it would seem that death may be associated with paradoxical embolism in 7–10% of cases of patent foramen ovale.

The association between major congenital heart disease and brain abscess has long been noticed. Farre records an instance in 1814, and Ballet, in 1880, drew attention to the association without guessing at its aetiology. It was Abbott, Lewis, and Beattie (1923) who first suggested that the occurrence of cerebral abscess in congenital heart disease might be due to paradoxical embolism, though the two cases they presented in support of this view are not altogether convincing. Hanna (1941) quotes 23 cases in which the conditions coincided, and Hand (1947) has collected

36 cases. It seems difficult to escape the conclusion that these abscesses arise as a result of paradoxical embolism. Further, in a number of cases (Bach, 1928 ; Rabinowitz, Weinstein, and Marcus, 1932 ; Wechsler and Kaplan, 1940) in which a cerebral abscess occurred in association with septal defects, no source of an embolus could be found ; it seems reasonable to explain these on the hypothesis that minute emboli from unobtrusive septic foci (which would normally lodge in the lungs without causing symptoms) may in the presence of a " right-to-left shunt " cause a cerebral abscess.

Although it is possible to collect a fairly large number of such cases from the rather extensive literature on the subject, the condition cannot be regarded as a common one. In Maude Abbott's (1932) review there were only seven examples of cerebral abscess. Gates, Rogers, and Edwards (1947), out of 115 fatal cases of major congenital cardiac anomaly, had five cases of cerebral abscess, but Brown (1939) in over 100 cases of Fallot's tetralogy had only one such abscess. In Pennybacker and Sellors's (1948) series of over 100 cerebral abscesses, no case was found which might have been caused in this way.

In the past there had been much discussion as to the transmission of material, other than a thrombus, from the right to the left heart through a patent foramen ovale. Zahn (1885) was the first to suggest that tumour metastases might be spread by paradoxical emboli, but neither his cases nor those of subsequent writers can be included on the careful criteria insisted on by Thompson and Evans (1930), and indeed their own case 5 cannot be accepted without reserve ; Willis (1948) and Walther (1948) regard it as a rare and unimportant event in the dissemination of tumours. Schmorl (1888) described paradoxical embolism of liver tissue in a case of traumatic rupture of the liver, and less convincing examples have been recorded by Lubarsch (1893) and Flexner (1896) in cases of hepatic necrosis. Abrikossoff (1913) reported a stillborn child with birth injuries in which there was an embolus of cerebellar tissue in a coronary artery, and Specht (1917) found a fragment of shrapnel in the left ventricle of a soldier with a widely patent foramen ovale, who died following a gunshot wound of the leg. The possibility of fat and air embolism occurring through an intra-auricular opening has also been discussed ; Fromberg (1913) has described cases of fat embolism of this type, and Steindl (1924) and Pamperl (1924) cases of air embolism, while the whole subject has been reviewed by Merkel (1934), but it must be confessed that the evidence presented in most of these cases is far from convincing.

Summary

Three cases of paradoxical embolism are recorded, in one of which there was a thrombus *in situ* in a patent foramen ovale ; 40 similar reports have been found, in which there was a clot *in situ* at necropsy. The mechanism of paradoxical embolism through the foramen ovale is discussed, and the various types reviewed.

Attention is drawn to the association between major congenital heart disease and cerebral abscess. It appears probable that such abscesses are caused by paradoxical embolism, sometimes by very small emboli from asymptomatic foci. The finding of an actual embolus that has travelled by this route is uncommon.

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NON-SILICIOUS ANISOTROPIC CRYSTALS IN TUBERCULOUS SALPINGITIS

BY

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Attention has recently been drawn to the similarity between the histological picture of silicious granuloma and tuberculosis of proliferative type in the Fallopian tube of man (Roberts, 1947). In the differential diagnosis emphasis has been placed on the value of the polarizing microscope. The purpose of the present paper is to draw attention to the presence of doubly refractile, but non-silicious, intracellular, crystalline material in two cases of salpingitis regarded as tuberculous. In one of the cases tuberculous endometritis has subsequently been revealed.

Case Reports

Case 1.—K.C., an unmarried woman of 22 years, complained of pain in the right iliac fossa of several hours duration. She denied any previous abdominal operation, curettage, or tubal insufflation. There was tenderness at the site of the pain, and this was also elicited on movement of the uterus.

At laparotomy on April 20, 1949, the right ovary was found to be enlarged and partly occupied by blood clot the size of a walnut. The right tube was swollen and infiltrated. Partial salpingo-oophorectomy was performed. The left tube and ovary appeared normal. A normal vermiform appendix was removed.

The patient continued to complain of pain in the lower abdomen for a few weeks after operation, but was discharged fit on May 30, 1949.

Pathology.—Of the three separate portions of the specimen, two consisted, respectively, of a narrow piece of ovary 1·5 cm. in length presenting a recently ruptured corpus luteum, and a mass of blood clot of the order of 2 cm. diameter.

The remaining portion of material comprised a segment of swollen Fallopian tube partly covered by loosely adherent blood clot. The fimbriae were not identified. On histological examination all sections of the tube showed a loss of the normal delicate villous architecture. The lumen was reduced in size and contained recent blood clot. The villi were greatly thickened. Within the mucosa, and limited to it, were numerous follicles composed of pale epithelioid cells with prominent multinucleate giant cells. Areas of confluent caseation were demonstrated in the centre of a few of the follicles but only after examination of numerous sections (Fig. 1). Around the follicles there was a wide zone of lymphocytes, and these cells, together with plasma cells, were numerous in the intervening stroma. The giant cells were of both Langhans's and foreign-body types. Within or adjacent to the latter, particles of translucent or pale yellowish material were frequent. These crystalline deposits occurred in aggregations of variable size, but were frequently of the order of 30 μ ; some were considerably smaller, others much larger. No constant crystalline form could be ascribed to them, but only occasionally did they appear acicular. They were strongly doubly refractile (Figs. 2 and 3). They were invariably soluble in dilute (N 1)

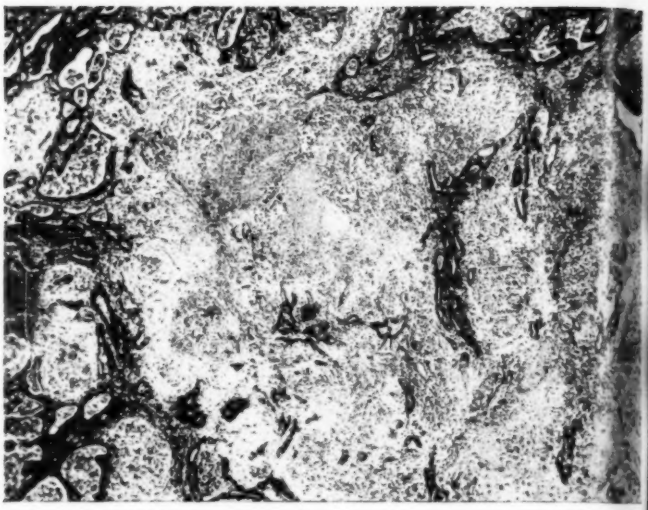


FIG. 1.—Section from Case 1 showing caseation. No laminated bodies or anisotropic crystals were present. (Haematoxylin-eosin. × 35).

mineral acids. In parts of the material they were accompanied by a different form of inclusion which appeared as a refractile, but not doubly refractile, concentric laminated body. These bodies were circular, dumb-bell or trefoil ; in size, widely variable but of the order of 50 μ. They were to a greater or lesser extent basophilic. Doubly refractile crystals were frequently closely associated with, or actually situated within, these bodies, which were themselves usually enclosed by foreign-body giant cells. Some, however, were extracellular and appeared to have ruptured the containing giant cell. No inclusions were seen in areas showing caseation. Despite painstaking search, acid-fast bacilli were not demonstrated in the numerous preparations examined.

Second Hospital Admission.—Fifteen months later the patient was re-admitted to hospital complaining of left lower abdominal pain and menorrhagia. Her uterus was small, anteverted, mobile, and displaced to the right side. The left tube and ovary were not thickened. X-ray examination of the chest and abdomen were negative. She was curetted on August 30, 1950. The histological picture was that of tuberculous endometritis. The material was cut in serial sections but no inclusions were found. During her stay in hospital the patient developed phlyctenular conjunctivitis.

Case 2.—F.B., a married woman of 22 years, complained of irregular periods associated with pain in the left iliac fossa. She had recently had premenstrual dysmenorrhoea and dyspareunia more to the left on deep penetration. She denied any previous abdominal operation or tubal insufflation but had undergone a curettage following a septic abortion in 1947. She had no family. The uterus was retroverted and pushed to the right, and a tender swelling was present in the left fornix behind the uterus.

At operation on September 27, 1949, a cystic swelling of about 3 cm. diameter was found in the recto-uterine pouch, with adhesions of cartilaginous density between uterus and rectum. Folds of ileum were adherent to the fundus of the uterus. The adhesions were separated and the cyst removed. The uterus was placed in anteversion and the round ligaments shortened.

She had an uneventful convalescence and was discharged fit on October 7, 1949.

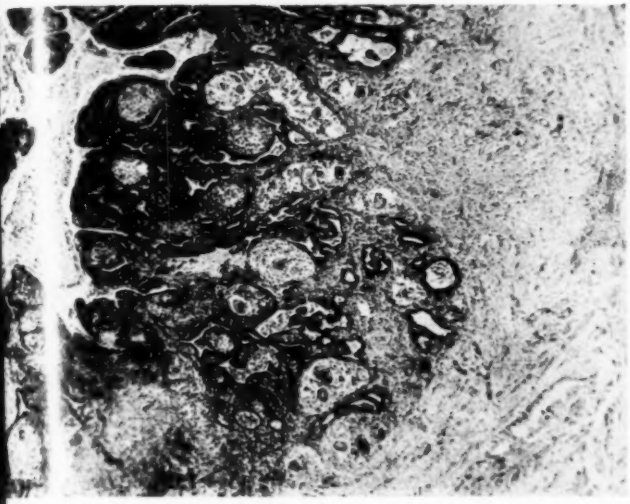
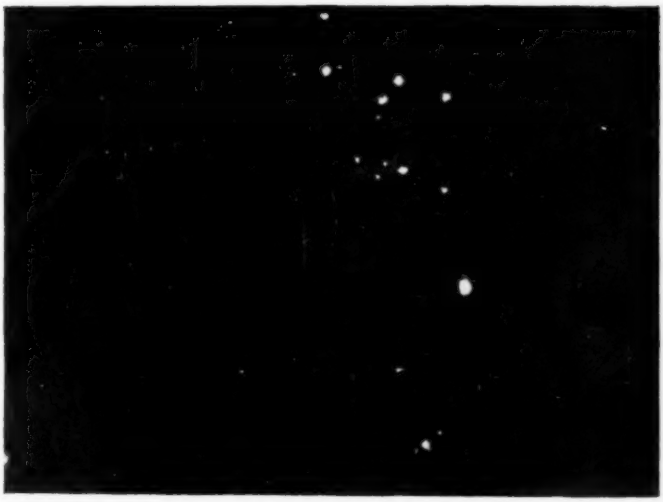


FIG. 2.—Section from mucosa of Case 1, lumen to left, photographed by ordinary illumination and through crossed polaroid screens. The numerous well-formed follicles show prominent giant cells. The doubly refractile crystalline material is inconspicuous by ordinary illumination. (Haematoxylin-eosin. × 30).



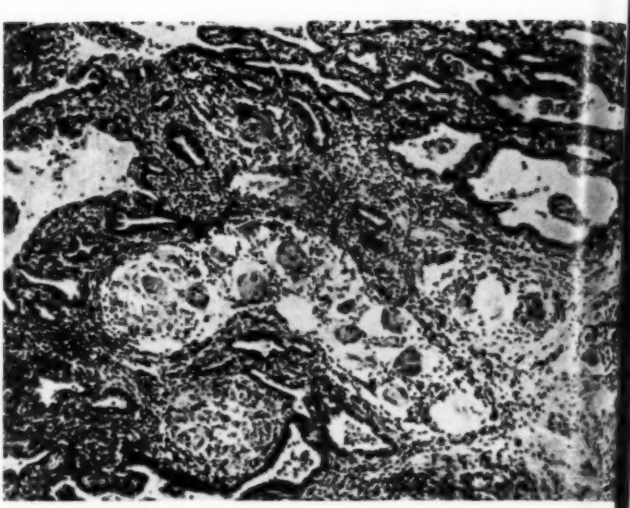


FIG. 3.—Section from mucosa of Case I photographed by ordinary illumination and through partially crossed polaroid screens. This is a higher magnification picture of the upper part of Fig. 1. No laminated bodies are present. All the doubly refractile material shown was acid soluble. (Haematoxylin-eosin. × 85.)

Pathology.--The specimen comprised three separate portions of material. One was a semicircular mass, 2 cm. diameter, consisting of blood clot presenting at one small area on its periphery a boundary zone of lutein cells supported by a narrow rim of fibrous tissue. The second portion consisted of fibrous and adipose tissue together with scanty unstriped muscle fibres. Many transverse sections of arterial wall were distributed throughout, and the tissue was presumed to be part of the broad ligament. Numerous lymph follicles were also present together with a patchy, but at places marked, infiltration by lymphocytes and plasma cells. In the more severely affected areas fibroblastic proliferation with newly formed capillaries was evident. No epithelioid cell follicles, giant cells, or doubly refractile material were present.

The remaining portion of material consisted of fused ovarian tissue and Fallopian tube. The ovarian part of this tissue showed at its peritoneal surface a granulomatous inflammatory lesion. Follicles of pale epithelioid cells were present together with multinucleate giant cells of both Langhans's and foreign-body types. In the granulation tissue intervening between the follicles lymphocytes and plasma cells were prominent, and a similar cellular infiltrate extended for some distance into the ovarian tissue, which was otherwise without significant features. This follicular granulomatous tissue reaction extended to a part of the serous surface of the tubal portion of the specimen. The subserous coat appeared thickened by fibrous tissue and the muscle partially replaced by similar tissue. In both these sites focal round-cell infiltration, mostly perivascular, was present. The lumen contained recent blood. The villi showed marked thickening and fusion, and the normal mucosal pattern was largely destroyed. The mucosa was beset with numerous follicles consisting of a central core of pale staining epithelioid cells with a poorly demarcated peripheral aggregation of lymphocytes and plasma cells: these cells were also prominent in the intervening stroma. Caseation was seen in occasional follicles around some of which concentric fibrosis had occurred. Areas of confluent caseation were scanty. In the greater part of the tube the follicles were non-caseous. Practically all follicles showed one or more multinucleate giant cells of both Langhans's and foreign-body types. The latter frequently, and the former infrequently, presented small intracytoplasmic crystalline inclusions of translucent or yellowish appearance inconspicuous by ordinary illumination but strongly doubly refractile. These crystals were invariably soluble in dilute mineral acids. A concentric laminated type of inclusion was also frequently encountered. These bodies of circular, trefoil or dumb-bell shape were of the order of 50 to 100 μ. Some were pale and inconspicuous by bright illumination, while others showed a strong affinity for haematoxylin and stained positively with the usual methods for demonstrating iron. They were not themselves anisotropic, but were frequently associated with the doubly refractile crystalline material (Fig. 4). These laminated bodies were usually within or adjacent to foreign-body giant cells, but were occasionally found lying free in the collagenous tissue further out in the wall. Tubercle bacilli were not found in numerous suitably stained sections.

Differential Histological Diagnosis

The tissue, in both instances, was received in neutral formalin fixative, and animal inoculation was therefore precluded. Although a careful search was made of numerous sections from each case, acid-fast bacilli were not demonstrated, a difficulty which is, however, widely recognized. The diagnosis rested, in each case, upon the presence of a granulomatous lesion having well-formed follicles, including numerous giant cells and frank caseation in some areas. It is emphasized that the original sections in the two cases showed no suggestion of caseation, and only in the examination of further material were follicles showing central caseation seen. Large areas of confluent caseation were seen in only a very few of the numerous sections.

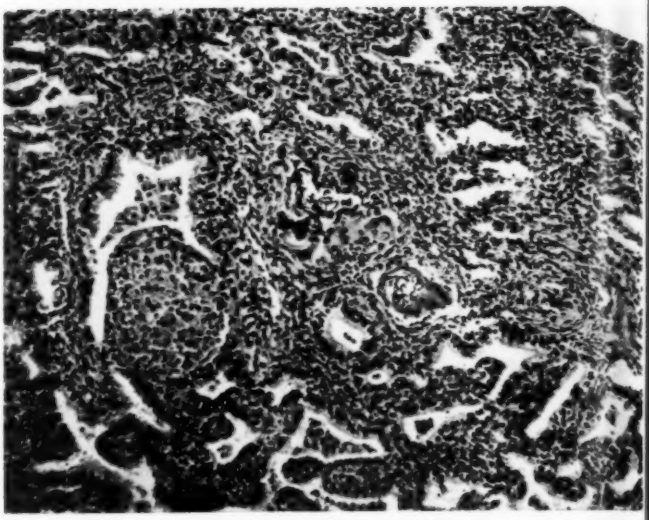
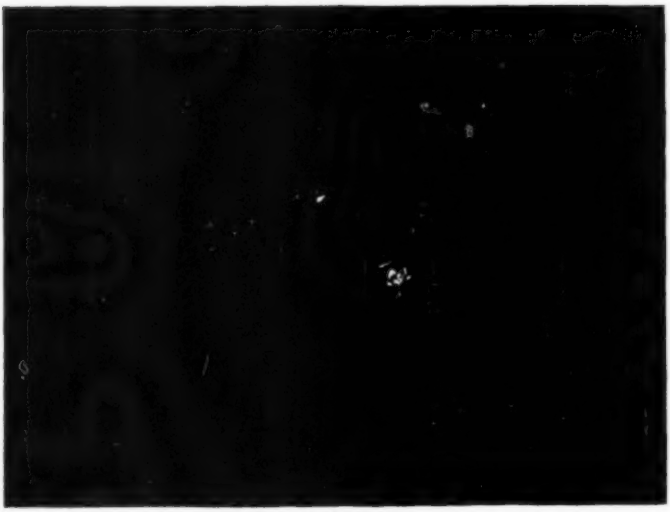


FIG. 4.—Section from Case 2 showing adenomatous pattern of mucosa and basophilic inclusion of trefoil shape. The intimate relationship of the crystalline material to such bodies is shown in the photograph of the same field through crossed polaroid screens. (Haematoxylin-eosin. × 115.)



It is notable, too, that inclusions were scanty or more commonly absent in relation to the caseous areas, and also that the multinucleate giant cells at the periphery of such areas were mostly of Langhans's type, while those in relation to the inclusions were usually of foreign-body type. Epithelioid cells were prominent in the follicles, whereas in silicious granuloma they are scanty or absent.

The Inclusion Bodies

The anisotropic crystals in each of the cases bore a close morphological resemblance to those of surgical talc. This similarity is emphasized by the frequent occurrence of rounded forms rather than spicules in silicious granuloma of the Fallopian tube (Roberts, 1947). Indeed, in the former of the two cases, the diagnosis of talc granuloma would probably have been made had I not been aware that there was no history of previous operation or other procedure to account for the presence of silica particles in the lesions. The solubility of the crystals in dilute acids established that they were non-silicious in nature: no doubly refractile material was found in numerous sections after acid treatment. The constant relationship of the inclusions to giant cells at once disposed of the possibility of artifact.

The concentric laminated bodies, though refractile, were not birefringent. They were nevertheless frequently closely associated with the anisotropic crystals, a fact that immediately suggested that their relationship was not coincidental. Indeed, many of the concentric bodies examined by polarized light revealed unsuspected enclosed crystals, usually in their centre. It is, however, worthy of note that these laminated inclusions were absent in some of the sections showing numerous crystals. Such a picture was presented in the original routine sections in the first of the cases here described, and the morphological resemblance to talc granuloma was correspondingly closer. Despite their size, the laminated bodies could readily be missed unless the illumination of the field was reduced. Some stood out prominently by virtue of their affinity for haematoxylin. A high proportion stained in whole, or in part, by the usual methods for iron.

It is not, however, the purpose of this paper to discuss the histochemistry of these inclusion bodies beyond the detail necessary to establish their identity with analogous bodies previously reported, and to which reference is made in the discussion to follow.

Discussion

The concentric laminated bodies correspond closely with those described and illustrated by several previous authors, of whom Berg and Bergstrand (1937), Schaumann (1941), Kraus (1942), and Teilum (1949) provide recent full accounts. Schaumann (1917) was the first author to report their presence in sarcoidosis, and they have in consequence been sometimes referred to as " Schaumann bodies." This eponymous term seems unfortunate, in that it tends to imply that they are specific for the sarcoid lesion. Ricker and Clark (1949) report that although they are not pathognomonic for sarcoidosis, they have not been observed in tuberculosis. Rich (1944), discussing their occurrence in sarcoid lesions, maintains that they are never encountered in " unequivocally tuberculous lesions in man or the ordinary laboratory animals." Moreover, bodies apparently entirely similar were described by at least 30 authors during the 60 years preceding Schaumann's description in 1917. Of these,

Bowen (1904), Hektoen (1902), Pelagatti (1901), Rona (1900), and Metchnikoff (1893), provide good descriptions. Lang (1875) is not infrequently accorded priority, but an excellent description and illustration of these bodies is contained in a monograph by Schüppel (1871) dealing with tuberculous lymphadenitis, and a good line drawing of them is provided by Billroth (1858). The earliest description which I have seen is that of Günsburg (1854), but Virchow (1864), in a published set of lectures in which mention is made of these bodies, refers to a previous report by himself in 1850. This last paper has proved inaccessible. Most of these early cases are concerned with their occurrence in lupus and tuberculous adenitis (some of which are clearly sarcoidosis), but more recent publications have noted their presence in pulmonary tuberculosis, leprosy, regional ileitis, and lymphopathia venereum. Pagel (1939), who reported their presence in the vole, referred to the bodies as " spheroid crystals." Many reports have referred to them as " laminated bodies," " peculiar bodies," or simply " inclusion bodies." In the absence of any commonly accepted name for these bodies, and to differentiate them from other named concentric laminated bodies occurring in the tissues, the term " corpora caepina " (onion bodies) is suggested as an appropriate descriptive designation.

So far as I am aware, this type of body has not previously been reported in the Fallopian tube. Bozzo (1945), however, recorded the presence of spherical " homogeneous and refrangent " bodies of 4–40 μ diameter in chronic salpingitis. The tissue reaction described was that of chronic non-specific inflammation, and no mention was made of multinucleate giant cells, nor was any illustration provided. In discussing the findings, he favoured the view that the inclusions were analogous to Russell bodies. Of the results of several staining methods, only an affinity for iron haematoxylin suggests an identity with the bodies described above. There is no reference to the use of specific stains for iron and no mention of crystals, anisotropic or otherwise.

It is remarkable that in an extensive search through the literature pertaining to laminated inclusions of the type described only two references have been found to the presence of associated doubly refractile material. Gottstein (1896) described the bodies in tonsillar tuberculosis and noted that they possessed a clear centre and that the " presence of lime salts is confirmed by examination with Nicol prisms." Skavlem and Ritterhoff (1946), referring to inclusion bodies in sarcoidosis, observed that " the colorless and yellowish tinged refractile material often enclosed by a Schaumann body is doubly refractile." These authors, however, do not make the point that such anisotropic material may greatly preponderate over the associated laminated bodies. While the relationship described by Skavlem and Ritterhoff was evident in many sections from the cases here presented, the fact that anisotropic crystalline material was present in some of the sections in which concentric laminated bodies were scanty or absent is a point of some importance. The occurrence of these anisotropic crystals in lesions of a follicular granulomatous type, which include numerous giant cells and may show no caseation, at once raises the possibility that the diagnosis of silicious granuloma might be reached erroneously in cases of tuberculous salpingitis. This is precisely the reverse of the very real pitfall to which Roberts has drawn attention. Moreover, the occurrence of two cases in which non-silicious anisotropic material was found in the Fallopian tube within a period of less than one year, during which time approximately 400 gynaecological specimens

of all types were examined, suggests that the presence of this material is not a rarity. It would therefore seem necessary to examine sections from several levels in the tube, not only, as Roberts suggests, in a doubtful case where the crystals are scanty, but in every case showing anisotropic material in relation to giant cells. The presence of laminated bodies in any part of a lesion presenting doubly refractile material must call for extra care in the interpretation of such crystals, although there is no reason why both non-silicious and silicious anisotropic crystals might not be present in a case of co-existent tuberculosis and silicious granuloma. The method of micro-incineration may provide information regarding the presence or absence of silica, but it is not readily applicable to routine work, and so far as these crystals are concerned the differentiation from silica may be made by treatment of appropriate sections with N/1 mineral acid for half an hour.

Summary

Two cases regarded as tuberculous salpingitis on histological evidence are described. Tuberculous endometritis was found subsequently in one of the cases.

The tubal lesions presented intracellular non-silicious anisotropic crystals and concentric laminated iron-containing bodies.

Comment is made on the unsatisfactory designations of these latter, and the descriptive term "corpora caepina" (onion bodies) is suggested.

The crystalline type of inclusion may greatly predominate or may occur alone, and in sections where caseation is absent the histological lesion closely resembles that of silicious granuloma.

The solubility of the crystals in dilute mineral acids is of differential diagnostic value.

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AN UNUSUAL CARDIAC ANEURYSM IN A YOUNG ADULT

BY

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It is generally agreed that the majority of cardiac aneurysms occur in elderly individuals following coronary occlusion. Aneurysms of the heart due to other causes are very rare and are seen with equal frequency at all ages. Most of these occur in the left ventricle, and, for all practical purposes, an origin from other parts of the heart can be neglected.

In the excellent review of the subject by Parkinson, Bedford, and Thomson (1938), the youngest case described was that of a woman of 42 years, who had an aneurysm at the apex of the heart. Aschoff nodules and an acute necrotizing arteritis with focal myofibrosis were found at necropsy. Burn, Hollander, and Crawford (1943) discussed the case of a girl of 13 years who had an aneurysm of the left ventricle in the region of the mitral ring. Although the lesions of active rheumatic fever were present, the authors considered a developmental weakness of the mitral valve was the most probable cause. Vivas-Salas (1948) demonstrated radiologically a ventricular aneurysm in a boy of 7 years which he believed was congenital in origin. An aneurysm of the ventricle in a man of 32 years which ruptured and caused death is described by Berlin and Hallen (1938). No evidence of coronary infarction was found, but the aneurysm appeared to arise in an area of myofibrosis, the aetiology of which was obscure. Three native boys of 6, 7, and 12 years respectively who had ventricular aneurysms are described by Macfie and Ingram (1920). The myocardium showed patchy fibrosis, but no specific aetiological factor was noted, although syphilis was considered a possibility in one case. Diverticula of the heart (Abbott, 1927 ; Roessler, 1944), which are usually found in infants, are sometimes described as aneurysms, but are only of academic interest. The other forms of cardiac aneurysms which have been described in the younger age groups are usually associated with anomalies of the coronary arteries (Bland, White, and Garland, 1933 ; Sanes and Kenny, 1934). We feel, therefore, that the following case of an aneurysm in a young adult is of interest, as the clinical and pathological findings were unusual, and we have been unable to discover a similar case in the English literature. Unfortunately Sternberg's (1914) monograph is not available in this country.

Clinical History

The patient, an unmarried Bantu woman aged 19 years, was admitted on May 10, 1950, to Baragwanath Non-European Hospital, complaining of a "throbbing" pain to the left of the sternum. A history of an operation for an abdominal swelling several

years previously was given, but no further information as to the exact nature of the lesion could be obtained. However, from details furnished by the patient, it was believed to have been an ovarian cyst. There was no history of rheumatic fever.

On examination a diastolic murmur was heard at the mitral area, but examination of the rest of the cardiovascular system showed no abnormality. As she had a raised temperature (99° F.), tachycardia, and an erythrocyte sedimentation rate of 30 mm. per hour (Westergren), a diagnosis of mitral stenosis and active rheumatic fever was made. The only abnormality in the chest seen radiologically (Fig. 1) was elevation of the left main bronchus which was interpreted as due to a dilatation of the left atrium. The serological tests for syphilis were negative.

Her condition improved on bed rest, and the temperature and E.S.R. fell, but a slight cough developed. She died unexpectedly 15 days after admission and death was considered due to acute cardiac failure.

FIG. 1.—Skiagram of the chest in left anterior oblique position. The elevation of the left main bronchus is visible, also the posterior bulging of the heart which was considered a dilated left atrium.

Post-mortem Findings

The body was that of a young Bantu female with a healed surgical midline incision in the lower abdomen. The fingers were not clubbed and only slight sacral oedema was observed.

The lungs (R. 500 g., L. 300 g.) showed moderate congestion and oedema, especially of the right lower lobe. On section there was congestion of the walls of the alveoli, some of which contained red blood cells and oedema fluid with a few haemosiderin-laden macrophages.

The hilar lymph nodes and spleen showed nodules varying from 1–4 mm. in diameter, which on section were confirmed as caseous tubercles.

The liver (1,400 g.) was dark red and blood oozed from the cut surface. On section the central veins and adjacent sinusoids were seen to be dilated, with compression and atrophy of the surrounding parenchymal cells. The histological features were those of subacute passive venous congestion.

The spleen (100 g.) was dark red and firm. The cut surface showed a few scattered necrotic nodules 1–5 mm. in diameter, which, on microscopic examination, showed the typical structure of caseous tubercles. In the surrounding splenic pulp the sinusoids were prominent and congested, but no evidence of increased fibrosis or chronic passive congestion was observed.

The kidneys (each 75 g.) showed scattered areas of cortical scarring, apart from which the cortex and medulla appeared normal with well-preserved vascular markings. No gross lesions were noted in the renal pelves or calyces. The cortex contained linear areas of interstitial fibrosis in which some of the glomeruli were partially sclerosed, with pericapsular fibrosis and atrophy of the corresponding tubules. Diffuse infiltration of these areas by lymphocytes and plasma cells was present. Some of the medium and small arteries showed a moderate degree of fibrous hyperplasia of the intima, but the majority were healthy. Slight perivascular lymphocytic infiltration and dilatation of the sub-epithelial capillaries of the pelves were noted, but, apart from passive congestion, the remaining cortex and medulla were healthy. The features were those of healed chronic pyelonephritis.

The left ovary and Fallopian tube were absent. It was considered that they had been removed surgically at the former operation.

Except for moderate congestion no pathological lesions were observed in the remainder of the gastro-intestinal and genito-urinary tracts. No other vascular lesions were found apart from those described in the heart.

The heart weighed 500 g. The pericardial sac was completely obliterated by fine fibrous adhesions. On examination a thin-walled sac (5 cm. in diameter) was observed arising from the region of the mitral ring on the postero-lateral surface (Figs. 2 and 3). In the upper portion of the sac there was laminated thrombus, the remainder containing blood. It communicated with the left ventricle by an opening (1 cm. in diameter) which was lateral to the mitral valve and unguarded by cusps. The aneurysm was superficial except for a common septum with the left atrium. The septum extended from the base of the heart to the mitral valve and was lined on the atrial side by endocardium and on the aneurysmal side by a thin layer of fibrin. Otherwise the atria were normal, with healthy appendices, and the foramen ovale was closed. The mitral valve was only 1.5 cm. in diameter, but the cusps and chordae tendineae were healthy. At the lateral commissure the cusps were continuous with the septum between atrium and aneurysm.

On the inferior surface the myocardium of the left ventricle merged into a band of fibrous tissue (3 mm. thick) which formed a gutter between the ventricle and the inferior cusp of the mitral valve (Fig. 4) and merged into the wall of the aneurysm.

The left ventricle showed only a slight dilatation, but the right was hypertrophied, the wall being 7 mm. thick. No abnormality was found in the aortic, pulmonary, and tricuspid valves.

Examination of the coronary arteries showed right coronary preponderance, the right artery supplying the posterior interventricular branch and the base of the heart as far as the left border. On macroscopic examination no lesions were observed in this vessel.

The first part of the left coronary artery and anterior interventricular branch were healthy. The circumflex branch, which was very small, narrowed 3 cm. from its origin and could only be traced with difficulty between aneurysm and ventricle to the left border of the heart, where it ceased.

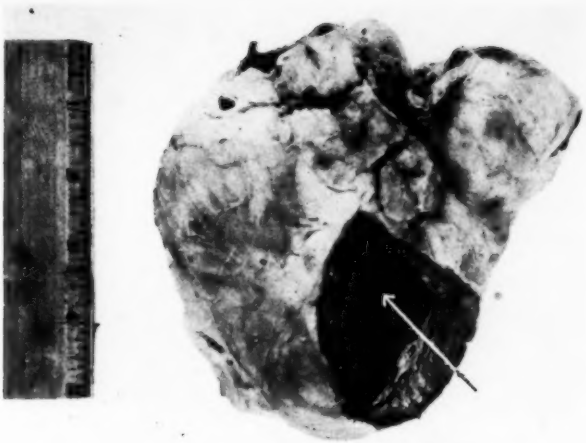

FIG. 2.—The aneurysm is observed in the region of the mitral ring. The left ventricle has been opened and contains a spigot (shown by an arrow) which passes directly into the sac. The left atrial appendix is lying between the aneurysm and the pulmonary artery.

FIG. 3.—The left side of the heart has been opened exposing the aneurysm and left atrium. The two halves of the aneurysm are seen separated with the atrium between them. The pointers lie in the two halves of the opening between ventricle and aneurysm.

No abnormality was present in the aorta and great vessels.

Microscopical Examination.—The wall of the aneurysm was composed of relatively acellular collagenous tissue. There were a few bands of cardiac muscle near the mitral ring, but they did not extend into the sac, whose wall was almost avascular apart from a few dilated capillaries on the epicardial surface. An endothelium was absent, and the sac was lined by a thin deposit of fibrin. The septum between atrium and aneurysm showed the same histological features, but the rest of the left atrial wall was normal. In the groove between the aneurysm and left ventricle the circumflex artery showed concentric fibrous hyperplasia of the intima with considerable diminution of the lumen. The internal elastic lamina was intact, but a few newly formed elastic fibres were observed in the thickened intima. The media was only slightly fibrosed. The left cardiac vein showed thickening and fibrosis of media and intima on the side adjoining the artery, but the remainder of the vessel wall was normal. The concentric hyperplasia of the intima did not suggest organization of a previous thrombus, and no evidence of active inflamma-

tory changes was found in the vessel wall or surrounding tissue. The histological features in the artery suggested a reactive endarteritis to some former irritant. In addition many of the small branches of the circumflex artery showed an obliterative endarteritis with almost complete obstruction. These changes in the small vessels extended to the inferior interventricular sulcus in the region of the fibrous gutter supplied by the right coronary artery. The major branches of the right and left coronaries elsewhere showed only patches of very slight intimal fibrous hyperplasia (Fig. 4).

The myocardium of the left ventricle was infiltrated by lymphocytes, mononuclear cells, and a few eosinophils extending diffusely between the muscle fibres (Fig. 5); an occasional focus was found with a perivascular distribution. No tubercle bacilli, spirochaetes, or other parasites were found by appropriate staining methods, but unfortunately culture was not performed at the time of the necropsy. In addition several foci of interstitial fibrosis associated with destruction of the muscle fibres were found, the largest being 2×2 mm. in diameter.

The gutter between the ventricle and the inferior mitral cusp was composed of hyaline collagenous tissue lined by a thin layer of fibrin. There were a few scattered bands of muscle fibres on the external surface.

The epicardial tissues were slightly infiltrated by scanty mononuclear cells and eosinophils, and the pericardial space was replaced by loose connective tissue.

In none of the many sections of the heart were Aschoff nodules found and no pathological lesions were observed in endocardium or valves.

The cause of death was attributed to acute right-sided heart failure due to obstruction of the mitral valve by a cardiac aneurysm.

Discussion

The narrowing of the mitral valve explained the presence of the murmur during life, the pyrexia being caused by either the myocardial or tubercular lesions. However, the pathogenesis of the aneurysm was more uncertain.

The following pathological conditions are listed by Parkinson, Bedford, and Thomson (1938) as the commoner causes of cardiac aneurysms.

Arteriosclerosis of the coronary vessels causes myomalacia and later fibrosis of the myocardium with resultant aneurysm formation. This is by far the commonest cause. Ten cases are described by Dressler and Pfeiffer (1940); 11 cases by Crawford (1943); 13 cases by Parkinson, Bedford, and Thomson (1938), but none of these occurred in the younger age groups.

An aneurysm secondary to a syphilitic gumma is rare, but cases are described (Sohval, 1935). One of us saw a multiloculated aneurysm in a male native of 39 years which was almost certainly of this nature.

Mycotic aneurysms are not uncommon, and may involve valve or myocardium. They follow infective endocarditis and are usually by themselves of little additional significance, but a case is described by Benson, Hunter, and Manlove (1933) in which rupture of such an aneurysm led to death.

Rheumatic aneurysms are believed to result from rheumatic necrosis, but are uncommon. Crawford (1943) states that "necrosis of the myocardium secondary to rheumatic lesions has been stated to be an aetiological factor but must be very rare." However, two of the cases mentioned in the introduction appear to be of

FIG. 4.—This shows the fibrous gutter between the left ventricle and atrium. The origin of the inferior mitral cusp can be observed at the upper border of the photograph. The right coronary artery is noted at the lower portion and the coronary sinus is seen to the right. (Masson × 7.5.)

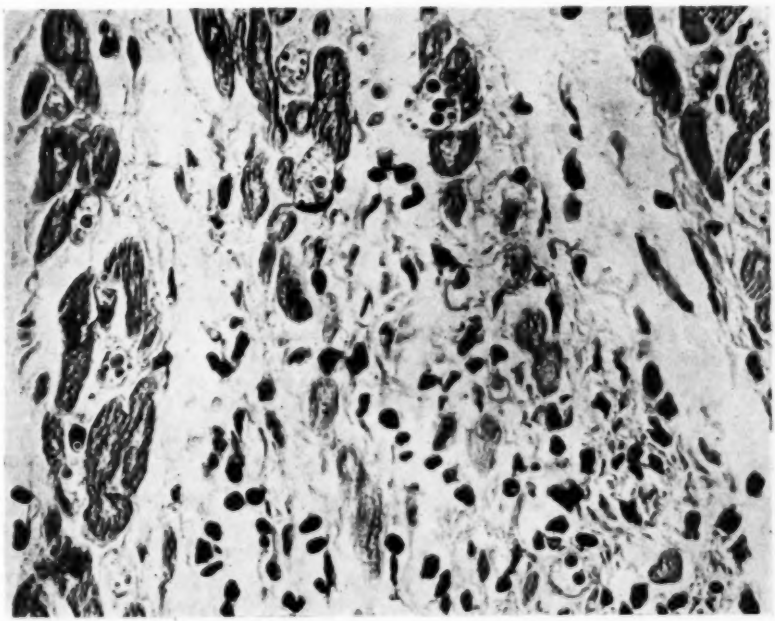

FIG. 5.—This section of the myocardium of the left ventricle shows infiltration by lymphocytes, monocytes, and a few eosinophils. In addition, some degenerating muscle cells are noted surrounded by the infiltrate. (Haematoxylin and eosin × 485.)

this nature (Parkinson, Bedford, and Thomson, 1938; and Burn, Hollander, and Crawford, 1943).

Congenital aneurysms are either diverticula of the heart or are related to the interventricular septum (Abbott, 1936). Other aneurysms described have been associated with the sinuses of Valsalva (Micks, 1940). Sometimes aneurysms related to anomalies of the coronary arteries are described as cardiac (Bland, White, and Garland, 1933).

Traumatic aneurysms are not common, but cases are described by Crawford (1943) and Thaon (1947).

In our case there was no history or pathological evidence of coronary thrombosis and infarction, and the important lesion appeared to be obliterative endarteritis in the small arteries and arterioles, only slight intimal thickening being apparent in the larger radicals of the coronary arteries.

Coronary thrombosis is very rare in the South African Bantu. Becker (1946) reported only one case in a pure Bantu in over 3,000 consecutive necropsies on coloured and Bantu subjects.

The lesions and clinical findings did not suggest syphilis and no evidence of bacterial endocarditis or traumatic damage was found.

It is possible that the aneurysm was due to a congenital weakness in the mitral ring, the vascular changes being secondary to pressure from the sac. However, the presence of the lesions at the base of the inferior mitral cusp and the generalized fibrosis and cellular infiltration of the myocardium renders this explanation improbable.

The obliterative endarteritis in the small arteries was not unlike that sometimes noted in chronic rheumatic endocarditis, but none of the other lesions found in this disease were observed, no lesions being present in the valves or endocardium and no Aschoff bodies in many sections. The nature of the myocarditis is uncertain. There was no clinical evidence of diphtheria and the degenerative myocardial changes appeared to be secondary to the cellular infiltration. A diffuse myocarditis is described very rarely in tuberculosis (Saphir, 1941), but in this case the histological changes in no way resembled this disease, and acid-fast bacilli were absent. As no other lesions were observed which afford an adequate explanation for the myocarditis, the case may be described as "isolated" or "Fiedler's" myocarditis. Isolated myocarditis in the South African Bantu does not appear to be uncommon, and we have already seen two other cases in 300 consecutive necropsies; in none was any cause observed.

Although secondary vascular lesions appear very rare in this condition, in this case we considered that they were secondary to the myocardial inflammation, postulating a sequence of events as follows. The obliterative changes in the small blood vessels led to a localized area of ischaemic fibrosis around the inferior part of the mitral ring. The aneurysm was then formed in this area in the same manner as those arising from an area of fibrosis after coronary occlusion.

Summary

A case of non-specific myocarditis and cardiac aneurysm is described in a young adult. The cause of the myocarditis and aneurysm could not be established with certainty.

A short discussion and review of aneurysms in young adults are given.

Our thanks are due to Dr. Vernon Wilson for permission to publish the clinical findings in this case, to Dr. B. J. P. Becker for invaluable criticism, and to Mr. F. A. Brandt for the photographs.

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THE INCIDENCE OF PENICILLIN-RESISTANT STAPHYLOCOCCI IN A SEMI-CLOSED COMMUNITY

BY

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Barber and Rozwadowska-Dowzenko (1948) recorded that naturally resistant penicillinase-producing staphylococci found within hospital communities had increased in the previous successive three years from 14% to 38% and then to 59%. Forbes (1949) isolated resistant staphylococci from 23 out of 50 nurses. Martin and Whitehead (1949) examined the nose, throat, saliva, and skin of 50 medical students and laboratory workers and found the carrier rate of resistant staphylococci to be 19.4% in this semi-closed community.

In view of the increasing incidence of resistant staphylococci in closed communities, it was decided to investigate the carrier state in another type of semi-closed community. The community chosen was a boys' grammar school. A constant set of 30 16-year-old boys occupying the same classroom had both anterior nares swabbed for the isolation of staphylococci. They were swabbed monthly over a period of 12 months.

The tube coagulase test was used to recognize *Staph. pyogenes*; strains were regarded as penicillin-resistant if their growth was inhibited by 0.25 to 0.5 units penicillin per ml. All resistant strains produced sufficient penicillinase after 18 hours' incubation to neutralize 30 units penicillin per ml.

Technique

The swab taken from both anterior nares was plated on to horse blood 2% agar (Table I A) on to 7.5% salt agar in the manner described by Maitland and Martyn in 1948 (Table I B), inoculated into nutrient broth (Table I C), and into broth containing 20 units penicillin per ml.

The blood and salt agar plates were incubated aerobically for 18 hours: the nutrient broths were incubated anaerobically for 18 hours and then subcultured on to blood agar plates for overnight aerobic incubation. The penicillin broths were incubated aerobically for 18 hours; no staphylococci were found in the tubes from this series, those with growth being found, by Gram stain, to contain Gram-negative bacilli.

All plates were left on the bench at room temperature for 24 hours in order to increase colonial pigmentation. Colonies believed to be *Staph. pyogenes* were tested for coagulase in 10% plasma broth to which heparin was added giving 2.5% Toronto units per ml. (Harper and Conway, 1948). All strains yielding clot within 18 hours were streaked on to penicillin ditch plates, each ditch containing 10 units penicillin per ml. After incubation the organisms growing up to the ditch were considered to be resistant

TABLE I

SUMMARY OF ALL STAPHYLOCOCCI ISOLATED OVER 12 MONTHS FROM THE SAME GROUP OF 30 SCHOOLBOYS

	A			B			C			
Month (1949)	No. of Colonies Picked	Coagulase Positive	S or R	No. of Colonies Picked	Coagulase Positive	S or R	No. of Colonies Picked	Coagulase Positive	S or R	No. of Carriers of Resistant Staphylococci
Feb. ..	33	14	14 S	29	16	15 S 1 R	11	7	7 S	6
Mar. ..	32	11	10 S 1 R	33	14	14 S	14	5	5 S	5
Apr. ..	41	13	13 S	40	17	15 S 2 R	11	11	10 S 1 R	6, 15
May ..	37	11	10 S 1 R	31	15	14 S 1 R	10	9	8 S 1 R	15
June ..	41	16	15 S 1 R	44	16	14 S 2 R	16	13	12 S 1 R	15, 19
July ..	40	13	11 S 2 R	44	17	14 S 3 R	16	13	11 S 2 R	6, 15, 19
Sept. ..	36	12	12 S	30	9	8 S 1 R	11	6	4 S 2 R	6, 15
Oct. ..	35	12	10 S 2 R	24	13	10 S 3 R	6	5	3 S 2 R	4, 10, 19
Nov. ..	30	8	8 S	26	8	8 S	4	3	3 S	None
Dec. ..	41	16	16 S	27	15	15 S	5	4	4 S	None
Jan. 1950	32	10	10 S	18	9	9 S	2	1	1 S	None
Feb. ..	31	9	9 S	14	6	5 S 1 R	2	1	1 S	19
Total ..	429	145	138 S 7 R	360	155	141 S 14 R	108	78	69 S 9 R	

A = Swabs direct on blood 2% agar plate. B = Swabs in 7·5% salt agar. C = Swabs in nutrient broth on to blood 2% agar plate. S = Sensitive to penicillin. R = Resistant to penicillin.

strains, and were tested for penicillinase production by their power to neutralize 30 units penicillin (Cruickshank, Squire, and Topley, 1948). The fluid broth culture was tested for complete penicillin neutralization on a staphylococcal pour-plate as described by Barber (1947).

The minimal bactericidal concentration of penicillin of these strains were determined, and sub-cultures were submitted to Dr. R. E. O. Williams for the determination and interpretation of the phage types.

TABLE II

DISTRIBUTION OF PHAGE-TYPED PENICILLIN RESISTANT STRAIN (*Staph. pyogenes*) IN SIX SCHOOLBOYS

Month (1949)	Boys Ref. No.	Phage Type									Percentage of Carriers
Feb. ..	6 B	52A									3·3
Mar. ..	5 A		6/47/47B (+)								3·3
Apr. ..	6 B			6/47/53 (+)							6·6
	15 B				6						
	15 C					6/47					
May ..	15 C						54 (+)				3·3
	15 B						54 (+)				
	15 A						54 (+)				
June ..	15 A							54 (VW)			6·6
	15 B				6						
	15 C							54 (VW)			
	19 B								7/47/47B/53/54 (+)		
July ..	6 B			6/47/53 (+)							10·0
	15 A						54 (+)				
	15 B						54 (+)				
	15 C						54 (+)				
	19 A								7/47/47B/53/54 (+)		
	19 B								7/47/47B/53/54 (+)		
	19 C								7/47/47B/53/54 (+)		
Aug. ..											
Sept. ..	6 B			6/47/53 (+)							Omitted
	6 C			6/47/53 (+)							6·6
	15 C						54 (+)				
Oct. ..	4 B									47/47B/53 (+)	10·0
	10 A										47/47B (+)
	10 B										47/47B (+)
	10 C										47/47B (+)
	19 A										
	19 B										
	19 C										
Nov. ..											
Dec.	Nil										Nil
Jan. 1950											
Feb. ..	19 B								7/47/47B/53/54 (+)		3·3

A, B, C = Technique by which the strain was isolated; see Table I.

Results

From Table I it can be seen that resistant penicillinase-producing staphylococci were isolated twice as often from the salt agar plates (B) compared with direct plating on to blood agar plates (A), and that preliminary broth inoculation (C) was slightly preferable to direct blood agar plating. Despite the fact that all resistant staphylococci isolated from A, B, and C neutralized 30 units of penicillin, none of these strains were recovered from direct inoculation into 20 units penicillin per ml. broth. All the resistant strains produced penicillinase.

On one occasion only was a resistant strain recovered from a blood agar plate (A) and not recovered by techniques B or C.

Table II enumerates the 10 different phage types isolated throughout the 12 months and their distribution between the boys.

There is no evidence of cross-infection between the boys, for at no time was an identical strain isolated from more than one individual.

Out of the 30 boys, a total of six carried resistant staphylococci at varying periods during the year. One boy, No. 6, was a carrier in February, April, July, and September: his strains in the latter three months of the year were identical but different from that found in February. Boy No. 15 carried one strain in April and June, another strain in May, July, and September, and two further distinct organisms in April and May, that is, he carried four varying phage types. Boy No. 19 carried one strain in June, July, and October, 1949, and February, 1950. Unrelated types of staphylococci were isolated once only from boys Nos. 4, 5, 10. On no occasion were there more than three carriers at a time.

None of the boys had septic lesions, nor, so far as could be ascertained, had they been in contact with staphylococcal infections in their homes.

Discussion

In a semi-closed community of 30 schoolboys, who were confined together for not less than six hours a day for five days a week, no evidence of cross-infection with *Staph. pyogenes* was obtained from nasal swabs taken at monthly intervals. Had the sampling interval been shorter it is possible that identical strains might have been found in a number of boys. It is evident that there is little likelihood of cross-infection in institutions which did not deal with sickness. In hospitals, where we know cross-infection prevails, Forbes (1949) found 12.5% resistant staphylococci in an open community from clinical sources of out-patients and 68.4% in in-patients. The resistant staphylococcal carriers were five times more common in in-patients than in out-patients.

Summary

A description is given of the isolation of resistant staphylococci from the nares of 30 schoolboys.

Ten different phage types were isolated from six boys.

There was no evidence of cross-infection. At no time throughout the 12 months did any one type of staphylococcus appear in more than one boy.

The highest carrier rate at any one time was 10%.

We should like to thank Dr. R. E. O. Williams for his help in determining and interpreting the phage types.

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THE AUREOMYCIN SENSITIVITY OF 100 PATHOGENIC STRAINS OF *STAPHYLOCOCCUS AUREUS*

BY

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Aureomycin, first described by Duggar (1948), has been used in the treatment of a wide range of bacterial infections. *Staphylococcus* has been listed as one of the more sensitive organisms, Bryer, Schoenbach, Chandler, Bliss, and Long (1948) finding that 0.6 μg./ml. was the smallest concentration of the antibiotic inhibiting growth in ordinary media. No figures were given for the number of strains tested, nor details relating to the source of these organisms. Evidence is available confirming the efficacy of this antibiotic in the treatment of staphylococcal bacteriaemia, especially in cases failing to respond to massive doses of penicillin (Ross, Burke, Rice, Schoenbach, Bischoff, and Washington, 1948). Localized skin infections have also responded to oral administration. It was decided, therefore, to investigate the aureomycin sensitivity of 100 pathogenic strains of *Staph. aureus*, obtained by culturing swabs from fresh septic lesions seen in the hospital casualty department.

Methods for the Isolation of Suitable Strains

One hundred and thirty-two swabs were examined in the course of this investigation. A short history accompanied each specimen, and only material from fresh septic lesions, seen for the first time in the casualty department, was accepted for culture. Swabs from patients already receiving antibiotics and from patients attending for daily dressings were discarded. On receipt in the laboratory, each swab was immediately inoculated on to three plates. One nutrient agar plate containing 5% sheep blood and one crystal violet horse blood agar plate (Garrod, 1933) were incubated aerobically. One 5% horse blood agar plate was prepared for anaerobic incubation. All aerobic plates were read after overnight incubation at 37° C. and again after standing for 24 hours at room temperature. Anaerobic plates were examined at the end of 48 hours' incubation. The number of colonies on the sheep blood plate was rarely less than 200 in the majority of swabs examined. Growth was frequently confluent at the site of primary inoculation and refractionation was occasionally necessary. The results of these preliminary investigations are set out in Table I.

Analysis of the information accompanying each specimen showed that the cases fell into the clinical categories enumerated in Table II.

One hundred organisms were selected from the first four groups after subjecting the strains to four accepted tests for pathogenicity.

1. Pigment Production.—The formation of pigment by strains of *Staph. aureus* was almost invariably apparent after overnight incubation at 37° C. In doubtful cases, where scraping up growth with a wire loop failed to demonstrate golden colour, standing at

TABLE I

RESULTS OF CULTURE OF 132 SWABS

Organisms Isolated	Number of Swabs Examined
Staphylococcus aureus	97
Staphylococcus aureus and *albus*	5
Staphylococcus albus	10
Coliforms	7
β-Haemolytic streptococcus and *Staphylococcus aureus*	1
Contaminants	4
No growth	8

TABLE II

CLINICAL CLASSIFICATION OF SWABS EXAMINED

Clinical Category	Number of Swabs Examined
Hand and finger infections	59
Axillary and breast abscess	14
Boils and carbuncles	16
Other small subcutaneous abscesses requiring incision	14
Pemphigus neonatorum	1
Miscellaneous minor sepsis	26
No diagnosis	2

room temperature for 24 hours always enabled a definite decision to be made. All strains used in the sensitivity tests produced abundant colour under these conditions.

2. Coagulase Production.—Two techniques were used.

Slide Technique.—The method described by Cadness-Graves, Williams, Harper, and Miles (1943) was used throughout as a screening test, and was subsequently confirmed by the tube technique. It was rapidly and conveniently performed and gave clearly defined results which were, with one exception, in accordance with the tube coagulase tests. The exception was a heavily pigmented strain of *Staph. aureus* isolated from swab No. 57. This was repeatedly negative by the slide method, despite the use of several samples of fresh plasma. In the tube, a firm clot formed after 30 minutes' incubation, and this was repeated on two occasions. All other strains in the series reacted vigorously on the slide.

Tube Technique.—Fresh human plasma diluted 1 in 10 was incubated in 1 ml. amounts together with 0.1 ml. of a broth culture of the organism under test, according to the method described by Fisk (1940). Gillespie (1943) recommends a similar technique which differs but slightly from this. Tubes were incubated at 37° C. and examined at hourly intervals for six hours. Many strains produced a firm clot whilst awaiting transfer to the incubator, and all those chosen for titration with aureomycin produced a clot in less than six hours.

3. Liquefaction of Gelatin.—It is generally accepted that the ability to liquefy gelatin cannot alone be taken as a reliable criterion of pathogenicity. Strains of *Staph. albus* frequently produce gelatinase, and in the course of examining 180 organisms Winslow, Rothberg, and Parson (1920) found that 47% of these reacted in this way. However, it is agreed that *Staph. aureus* liquefies gelatin more frequently and more rapidly than *Staph. albus*, and taken in conjunction with other tests, this property can be useful in the assessment of pathogenicity.

In the present series, gelatin stab preparations were incubated at 25° C. for five days. All selected strains produced saccate or infundibuliform liquefaction within this period. Organisms which failed to produce alteration in the medium after five days were discarded.

4. Fermentation of Mannitol.—Opinions differ widely regarding the value of biochemical activity in assessing the pathogenicity of staphylococci. As with the production of gelatinase, it is generally believed that *Staph. aureus* is more active and *Staph. albus* less so. Dudgeon and Simpson (1928) found that strains of *Staph. aureus* almost invariably fermented mannitol, whilst *Staph. albus* frequently did so. The rapidity with which mannitol is fermented by various strains has been employed in a method for grading pathogenicity (Knott and Blaikley, 1944). It was therefore decided to exclude all staphylococci which failed to ferment mannitol after overnight incubation at 37° C. Many of the strains were seen to have fermented the sugar at the end of six hours.

Strains of *Staph. aureus* considered suitable as a result of the above tests were subcultured on to Dorset's egg medium and stored at room temperature after preliminary incubation.

Preparation of Aureomycin Solutions

Aureomycin hydrochloride was used throughout the investigation to prepare suitable solutions for sensitivity titrations. This substance is a yellow, crystalline solid, readily soluble in distilled water and to a less extent in saline. Owing to the high bulk-weight ratio, the salt can be easily weighed out in 1 mg. quantities on to a sterile watch-glass, using a micro-balance. Solutions of the salt in water have a *p*H of 4.5. When made alkaline, these solutions rapidly lose antibacterial activity. It was clear, therefore, that normal liquid media, with an approximate *p*H of 7.4, could not be used as solvents for the dry hydrochloride. Chandler and Bliss (1948) found that the rate of deterioration of aureomycin solutions was fairly constant for any given test organism. They maintained bacteriostasis during testing by adding fresh amounts of drug every 24 hours. As an alternative, it was decided to investigate the properties of solutions adjusted to a lower *p*H which would not inhibit the growth of the test organism. Staphylococci inoculated into glucose broth adjusted to levels below *p*H 6.0 showed evidence of delayed growth when compared with cultures in normal broth. At *p*H 6.8 growth was vigorous, and tryptic digest broth containing 0.25% dextrose was adjusted to this level for the initial experiments to determine the stability of aureomycin solutions.

Quantities, each of 1 mg. of aureomycin hydrochloride, were allowed to dissolve in one or two drops of glucose broth, then transferred by Pasteur pipette to 100 ml. of broth. The residue on the watch-glass was taken up with several more drops of broth and added to the container, thus giving a solution with a final concentration of 10 μg./ml. It was rather more yellow than normal broth, but the final *p*H was not altered from 6.8 by the addition of the antibiotic. Using 1 ml. as unit volume, doubling dilutions of this solution in glucose broth were made in 3 in. $\times \frac{1}{2}$ in. tubes, to a total of 10 tubes. One tube, containing glucose broth at *p*H 6.8 without aureomycin, was included in each rack as a growth control. One strain of *Staph. aureus*, cultured from swab No. 106, was selected as a test organism. To each tube 0.02 ml. of a six-hour broth culture was added from a dropping pipette and the rack incubated at 37° C. for 18 hours. At the end of this period, a reading was made by observing the presence or absence of diffuse, uniform turbidity. After a number of trials, it was found that the test organism was consistently inhibited by an apparent concentration of 0.625 μg./ml. The end-point was clear and well defined. However, it was obvious that the solution might have lost potency either whilst standing at room temperature for varying periods of time or during incubation at 37° C. It was necessary, therefore, to examine solutions kept at room temperature. A container of 100 ml. of glucose broth containing 10 μg./ml. of aureo-

mycin was left at room temperature (21° C.) and samples withdrawn at hourly intervals for a total of six hours. Each sample was titrated with strain 106 and transferred to the incubator immediately afterwards. It will be seen from Fig. 1 that the total age of each sample was 24 hours, compounded at varying times at 21° C. and 37° C. The first sample was titrated at once and was incubated for 24 hours at 37° C. The final sample remained at room temperature for six hours and was incubated for 18 hours. All racks were read the following morning and no variation from the previous recording of 0.625 μg./ml. was observed.

A further 100 ml. of aureomycin solution of similar strength was prepared and divided into two 50-ml. quantities. One portion was left at room temperature (21° C.) for a total of six hours, and titrated at hourly intervals as before. The other 50 ml. quantity was placed in the incubator at 37° C. for the same period, and also titrated hourly. The results were similar to those shown in Fig. 1. The observed sensitivity of strain 106 showed no variation from 0.625 μg./ml. in either sample. It was therefore considered that aureomycin solutions prepared in this manner possessed sufficient stability to warrant their use in the proposed sensitivity tests. At present, little is known of the way in which aureomycin produces its effect *in vivo* and *in vitro*. In the course of bacteriological studies, Price, Randall, and Welch (1948) noted a marked variation between the bacteriostatic and bactericidal end-point. However, they recorded the fact that destruction of the aureomycin was doubtless occurring during the incubation period, since they were using solutions at *p*H 7.0 and above. They found that various media ingredients caused marked destruction, and that thioglycollate medium tended to preserve the potency of the aureomycin to a greater degree than other media used. In the present series it would appear that, for the staphyloccoccus strain 106, aureomycin solutions at *p*H 6.8 retain those properties which enable consistent *in vitro* readings to be made. Whether sensitivities so obtained bear any relationship to the effective blood-level during treatment of staphylococcal infections is a matter for additional investigation. Furthermore, it is not yet determined whether this method of testing sensitivity is applicable to other organisms.

Sensitivity Testing of Pathogenic Strains

Titrations were carried out in the manner described, 12 strains being tested each day. Organisms were transferred from Dorset egg slopes into 5 ml. quantities of nutrient broth. Subculture from broth on to segments of blood agar plates was made in every case to check purity. Racks were read after 18 hours' incubation at 37° C.

Results

Fig. 2 analyses the results of 100 strains tested under these conditions. It will be seen that 81 organisms were sensitive to 0.625 μg./ml. of aureomycin or below. Nineteen strains were inhibited by 1.25 μg./ml. The lowest effective concentration in the series was 0.078 μg./ml.

Summary

One hundred pathogenic strains of *Staph. aureus* were isolated from fresh septic lesions seen in the hospital casualty department.

A method is described for the preparation of aureomycin solutions which appear to possess considerable stability when titrated with strains of *Staph. aureus*.

The aureomycin sensitivity of the collected strains, determined by this technique, is recorded.

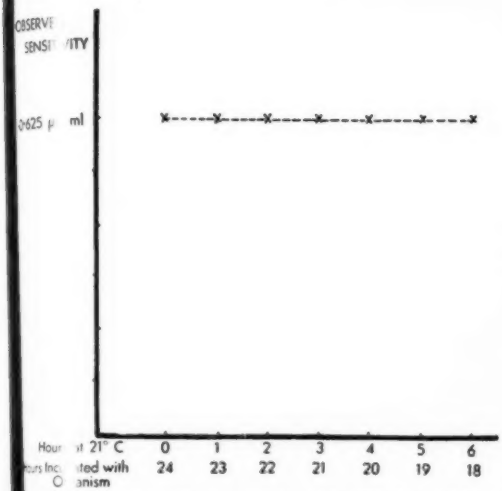

FIG. 1.—Titration of aureomycin hydrochloride solution kept at room temperature (21° C.) for six hours.

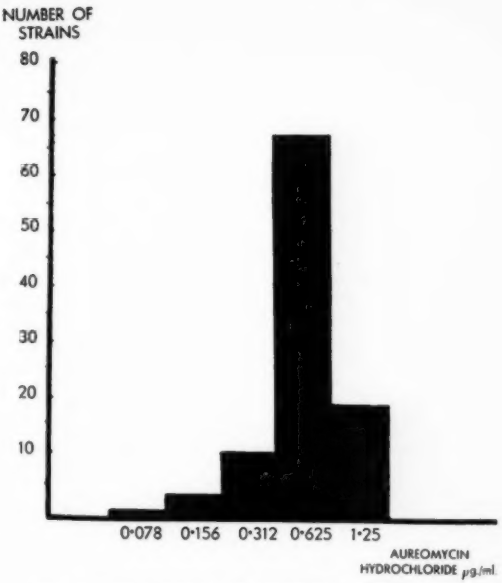

FIG. 2.—Distribution of sensitivity of 100 strains of *Staph. aureus* to aureomycin hydrochloride.

I am indebted to Professor R. Knox for his advice and encouragement during the preparation of this paper; to Mr. Patrick Clarkson for permission to collect material from the Casualty Department, and to the Department of Medicine, Guy's Hospital, for supplies of aureomycin hydrochloride. My thanks are also due to the casualty officers on duty for their co-operation in obtaining the material for this investigation.

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THE ROLE OF SPERMINE IN THE INHIBITION OF *STAPHYLOCOCCUS AUREUS* BY HUMAN SEMEN

BY

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The inhibitory activity of human semen on the growth of *Staphylococcus aureus* has been reported previously (Rozansky, Gurevitch, Brzezinsky, and Eckerling, 1949).

The Function of Human Semen

The investigation has now been extended using 12 additional freshly isolated strains of *Staph. aureus*.

Materials and Methods.—Seventy-five specimens of semen from 53 patients (of the male sterility clinic) between the ages of 23 and 51 years were examined. Six of these were azoospermic, 25 oligospermic (less than 60 million spermatozoa per ml.), and 22 were normospermic. Ten specimens of human blood serum, 10 of cerebrospinal fluids, 10 of fluids from ovarian cysts, eight of amniotic fluids, four of pleural exudate, three of tears, and three of ox semen* were also tested in a similar manner. Materials were only taken from patients who had not received antibiotic treatment. The specimens of human semen were kept at room temperature for four hours before examination, then tested immediately, or after 72 hours at 8° C. or 14 days' refrigeration at 8° C. Twelve specimens were also examined after heating at 90° C. for 30 minutes. In addition to the three strains of *Staph. aureus* used in the previous study, 12 freshly isolated strains from surgical cases or pyogenic skin infections were tested. All strains were actively haemolytic, coagulase-positive, and mannitol-positive. The method described in the previous study (Rozansky *et al.*, 1949) was used. On plates inoculated with the test strain of *Staph. aureus*, six cups with an external diameter of 8 mm. were placed. Each cup received one or two drops of the fluid to be tested, the plates were incubated overnight at 37° C., and the zone of inhibition measured. Wherever possible, two examinations of each specimen were made. Difco nutrient agar of *p*H 6.8, used throughout the previous experiments, was employed at the beginning of this series, but it was later found that a slightly alkaline medium gave better results. The remaining tests were performed on agar plates of *p*H 7.4.

Results.—As may be seen from Table I, of the 41 specimens of semen examined, 37 inhibited the growth of the Heatley strain of *Staph. aureus* over an area 10–25 mm. in diameter. Of these 37 specimens, 14 were normospermic, 21 oligospermic, and two azoospermic. Nineteen of these specimens were tested in duplicate and similar

* Obtained by courtesy of Dr. Y. Flesh, Jerusalem, and Mr. R. Mentzel, Israel Cattle Breeders' Association centre for artificial insemination, Kinereth.

TABLE I

INHIBITION OF THE GROWTH OF *Staphylococcus aureus* (HEATLEY STRAIN) BY HUMAN SEMEN

	No. of Specimens Tested	Positive	Negative
Normospermic	17	14	3
Oligospermic	21	21	0
Azoospermic	3	2	1
Total ..	41	37	4

Semen tested after 4 hours' storage at room temperature.
Positive = Area of inhibition 10–25 mm. in diameter.
Negative = No inhibition.

areas of inhibition were found on both plates. Four specimens (three normospermic and one azoospermic) showed no area of inhibition when first examined, i.e., four hours after the collection of the material, but one of these retested after 72 hours produced an area of inhibition 13 mm. diameter on two plates. The other three specimens could not be examined a second time. Seven specimens heated for 30 minutes at 90° C. all retained their inhibitory effect on the growth of *Staph. aureus.*

Table II summarizes the results obtained with 21 specimens after 72 hours' cold storage. Twelve specimens were of the series already tested four hours after collection, and nine were tested for the first time after 72 hours' storage. Of the 21 specimens, 20 (eight normospermic, ten oligospermic, and two azoospermic) gave positive results, 15 of them tested in duplicate, and six on one plate only. Four of these specimens were heated at 90° C. for half an hour. The results showed no change in their inhibitory activity.

TABLE II

SEMEN TESTED AFTER 72 HOURS' STORAGE AT 8° C.

	No. of Specimens Tested	Positive	Negative
Normospermic	9	8	1
Oligospermic	10	10	0
Azoospermic	2	2	0
Total ..	21	20	1

Table III summarizes the results with 12 specimens after 14 days' storage in the refrigerator. Eight specimens (three normospermic, three oligospermic, and two azoospermic) were found to be positive, and four (one normospermic and three oligospermic) negative. Three of the specimens giving negative results had been positive when initially examined four hours after the collection of the specimens.

Table IV shows the results of the inhibitory effect of human semen on 14 different strains of *Staph. aureus.* One hundred and twenty-four tests were made with 75 specimens of semen. Strains No. 598 and No. 924, examined at the beginning of the present study, were tested with agar plates of *p*H 6.8 which may account for the greater number of negative results obtained.

TABLE III

SEMEN TESTED AFTER 14 DAYS' STORAGE AT 8° C.

	No. of Specimens Tested	Positive	Negative
Normospermic	4	3	1
Oligospermic	6	3	3
Azoospermic	2	2	0
Total ..	12	8	4

TABLE IV

INHIBITORY EFFICACY OF HUMAN SEMEN ON 14 FRESHLY ISOLATED STRAINS OF *Staphylococcus aureus*

Staph. aureus Strain	No. of Specimens Tested	Positive	Negative	Media
598	9	5	4	*p*H 6·8
924	7	4	3	,, ,,
1498	6	5	1	*p*H 7·4
1605	7	5	2	
1705	1	1	0	
1718	3	3	0	
2191	14	14	0	
2196	3	3	0	
2315	2	2	0	
2528	6	6	0	
2200	5	4	1	
750	4	4	0	
314	4	4	0	
2201	4	3	1	
Totals 14	75	63	12	

Positive = Area of inhibition 12–25 mm.
Negative = No inhibition.

Starting with *Staph. aureus* strain No. 1498, agar plates of *p*H 7.4 were used, and on alkalinized agar 54 specimens were positive and only five gave negative results, with areas of inhibition of the freshly isolated strains similar to those obtained with the Heatley strain. The three specimens, after cold storage for 21 days, tested with *Staph. aureus* No. 1718, gave positive results. From these results it is obvious that freshly isolated strains of *Staph. aureus* are as readily inhibited as the Heatley strain which has been used in our laboratory for several years.

Discussion

As the inhibitory effect of semen on the growth of *Staph. aureus* was observed both in normospermia and azoospermia, the active principle cannot be related to the presence of spermatozoa but is contained in the fluid portion of the semen. That the active principle is thermostable was shown by 12 specimens of semen

which, after being kept at 90° C. for half an hour, were tested with one or more strains of *Staph. aureus*; all retained their inhibitory activity.

Three specimens of ox semen were tested using four strains of *Staph. aureus*. Twelve examinations were performed, all of which gave negative results. Since the *p*H of ox semen is 6.6–6.8, four additional tests were made after alkalinization to a *p*H of 7.6–8.0 in order to approximate it to the *p*H of human semen. However, these alkalinized ox semen specimens also gave negative results.

Serum, tears, amniotic fluid, cerebrospinal fluid, pleural exudate, and fluid from an ovarian cyst were tested in a similar manner and did not show any signs of inhibitory activity. Four specimens of semen were tested against *Esch. coli, Salm. paratyphi A, Ps. pyocyanea,* and *Bact. alkalescens*, and, as previously reported, showed no evidence of inhibition.

The Hypothetical Function of Spermine and Spermidine

In view of the thermostability of the growth-inhibitory agent it was concluded that this agent was not a protein. It was assumed that the poly-amines, spermine and spermidine, found in human semen might be responsible for the inhibition of bacterial growth. Inhibition of the growth of *Staph. aureus* by spermine was observed by Bichowsky-Slomnitzki (1948). Synthetic spermine tetrahydrochloride and spermine phosphate extracted from human semen were therefore tested for their inhibitory effect on the growth of *Staph. aureus*.

Materials and Methods.—Spermine tetrahydrochloride (La Roche) was dissolved in distilled water in dilutions varying from 12.5 mg.% to 200 mg.%. Spermine phosphate was extracted from pooled human semen, according to the method described by Harrison (1933). Each of three pooled samples, obtained from 20 ml. of semen, yielded about 5 mg. of crystalline spermine phosphate. Since spermine phosphate is readily soluble in slightly alkaline solutions (Guggenheim, 1940), a 200 mg.% solution was made in distilled water by adding n/100 NaOH till *p*H 7.4 was reached.

The inhibitory activity of spermine tetrahydrochloride was tested on the Heatley strain and five freshly isolated strains of *Staph. aureus*, and spermine phosphate was tested on the Heatley strain and three of these other strains. All the strains were actively haemolytic, coagulase-positive, and mannitol-positive.

The method used was that described above, using Difco nutrient agar of *p*H 6.8 and the same agar alkalinized to *p*H 7.4 by adding a few drops of n/10 NaOH.

Results.—Using alkalinized agar (*p*H 7.4), spermine tetrahydrochloride (Table V) in a concentration of 200 mg.% inhibited the bacteria in all the tests. A concentration of 100 mg.% inhibited the growth in 43 of 44 tests, and a concentration of 50 mg.% inhibited the growth in 24 of 27 tests. Using a concentration of 25 mg.%, it inhibited 17 of 22 tests, but with a concentration of 12.5 mg.% it inhibited only five of 13 tests. Using the same concentration in an agar of *p*H 6.8, 24 of 25 tests were negative and only one test, with a concentration of 100 mg.% spermine tetrahydrochloride, was positive.

Three specimens of spermine phosphate extracted from pooled human semen were similarly tested (Table VI). Twelve tests using a concentration of 200 mg.%, and nine tests with a concentration of 100 mg.% on agar of *p*H 7.4 all gave positive results, the area of inhibition being 11–18 mm. in diameter. With agar of *p*H 6.8, of eight

TABLE V

INHIBITION OF SIX STRAINS OF *Staph. aureus* BY SPERMINE TETRAHYDROCHLORIDE (LA ROCHE)

Concentration (mg. %)	Medium *p*H 7·4			Medium *p*H 6·8		
	No. of Tests	Positive	Negative	No. of Tests	Positive	Negative
200	10	10	0	4	0	4
100	44	43	1	8	1	7
50	27	24	3	7	0	7
25	22	17	5	4	0	4
12·5	13	5	8	2	0	2

Positive test = Area of inhibition 11–18 mm. in diameter.
Negative test = No inhibition.

examinations, six were positive (area of inhibition 10–14 mm. in diameter), and only two negative.

Discussion

The inhibitory activity of spermine tetrahydrochloride is influenced by the *p*H of the agar. Only very high concentrations of spermine tetrahydrochloride were able to inhibit the growth on agar of *p*H 6.8. A concentration of 1,000 mg.% of spermine tetrahydrochloride was tested twice, an area of inhibition up to 22 mm. being found on agar of *p*H 7.4, and an area of 18 mm. on agar with *p*H 6.8. With extracted spermine phosphate, the effect of the *p*H of the medium was not as striking and the results obtained resembled the findings with human semen, where greater areas of inhibition were obtained on alkaline agar of *p*H 7.4 than on agar of *p*H 6.8. As reported above, three specimens of ox semen of *p*H 6.6–6.8, and the same specimens alkalinized to *p*H 7.6 and 8.0, had no inhibitory effect on the growth of *Staph. aureus*. Since ox semen does not contain spermine (Guggenheim, 1940 ; Harrison, 1931) this furnishes additional evidence of the role of spermine in inhibiting the growth of *Staph. aureus* by semen. The concentrations of spermine tetrachloride and spermine phosphate used in our experiments were within the range of the spermine phosphate concentrations found in human semen, i.e., 60–250 mg.% (Guggenheim, 1940 ; Harrison, 1933). The results of the present study seem to warrant the conclusion that spermine is the principle active in the inhibition of bacterial growth by human semen plasma.

The role of spermidine, the second poly-amine present in human semen, has not yet been examined.

Spermine tetrahydrochloride, spermine phosphate, and plasma of human semen showed no inhibitory activity when tests were performed with Bacto stock culture agar (*p*H 7.5).*

* A medium of a more complete nature than our standard medium. Apparently one or more of its components counteract the antibacterial effect of spermine.

TABLE VI

INHIBITION OF FOUR STRAINS OF *Staph. aureus* BY THREE SPECIMENS OF SPERMINE PHOSPHATE EXTRACTED FROM POOLED HUMAN SEMEN

	Medium *p*H 7·4			Medium *p*H 6·8		
Concentration (mg. %)	No. of Tests	Positive	Negative	No. of Tests	Positive	Negative
200	12	12	0	5	4	1
100	9	9	0	3	2	1

Positive test = Area of inhibition 11–18 mm. in diameter.
Negative test = No inhibition.

Positive test = Area of inhibition 10–14 mm. in diameter.
Negative test = No inhibition.

Summary

Human semen, synthetic spermine tetrahydrochloride, and spermine phosphate extracted from human semen have been examined for their inhibitory effect on *Staphylococcus aureus*.

Human semen inhibited the growth of the 15 strains of *Staph. aureus* examined, the inhibition zone being larger on alkaline agar.

Spermine tetrahydrochloride, in concentrations of 100 mg.% and over, inhibited the growth of *Staph. aureus* on alkalinized media (*p*H 7.4) in 53 of 54 tests performed. Using the same medium and concentrations spermine phosphate inhibited the growth in all 21 tests performed. Spermine tetrahydrochloride, in concentrations up to 200 mg.%, was active only if the medium was alkaline (*p*H 7.4). Spermine phosphate in that concentration was active both when the medium was alkaline (*p*H 7.4) and also, although to a lesser degree, when the medium had a *p*H of 6.8. In this respect spermine phosphate resembles the plasma of human semen.

It is evident that the inhibition of the growth of *Staph. aureus* by human semen is due to its spermine content, though other substances which have not been examined may play a part.

We are indebted to B. Shapiro, Ph.D., and to E. Margoliash, M.D., for their helpful advice.

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TECHNICAL METHODS

ULTRAFILTRATION OF PLASMA URIC ACID

BY

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In view of the possibility that the uric acid of the blood might be partly "bound" to protein and partly "free" (Benedict and Behre, 1931) and that this might provide some explanation for the phases of gout, it was decided to investigate various methods of ultrafiltration of the plasma uric acid in gouty and normal individuals.

Lambie (1940) reported a case of leukaemia with subsequent development of gout in which the ultrafiltrable fraction of the plasma uric acid was only 28%, but changed after splenectomy to normal, i.e., 100%. Lambie used collodion membranes for the ultrafiltration. His explanation was a change in the colloidal state of the blood urates.

Adlersberg, Grishman, and Sobotka (1942) reviewed the literature on the solubility of uric acid, reporting Minkovski's view on the free and bound uric acid in body fluids and the different results hitherto gained by ultrafiltration of serum and plasma and the explanation given by the different authors. Adlersberg *et al.* ultrafiltered serum, using 80 lb. nitrogen per sq. in. (positive pressure) and a "600" cellophane membrane for 18 to 24 hours. They reported that in normal individuals 76–96% (average 84%) of the uric acid was filtrable. They claimed further that, in contrast to normal individuals, gouty patients show a considerable fluctuation in the uric acid partition, even when the total blood uric acid level remains constant. In three out of 10 gouty patients 80% was the average.

Wolfson, Levine, and Tinsley (1947) used the anaerobic ultrafiltration method of Lavietes (1937), and found in normal individuals (13 filtrations in two patients) an ultrafiltrability of plasma uric acid of 79%. They used "600" cellophane membrane, which they soaked for three days at 0° C. before use.

Our first attempt was made with the positive pressure method of Adlersberg *et al.*, but we found that we could not filter more than 20–30% of the uric acid, possibly because of shortcomings in our filtration arrangements. We therefore adopted the method of Lavietes, used by Wolfson and his colleagues, which is as follows.

Method

Two pyrex half-cells (Fig. 1) blown from capillary tubing of 1.5 mm. internal bore 25 mm. deep, with rims ground to form an accurately fitting junction, were used. Between the two half-cells the soaked cellophane membrane was placed and held by pressure applied by a clamp and an elastic band. One limb (1) of half-cell A (Fig. 2) was connected by approximately 40 mm. long pressure tubing to a bent capillary tube

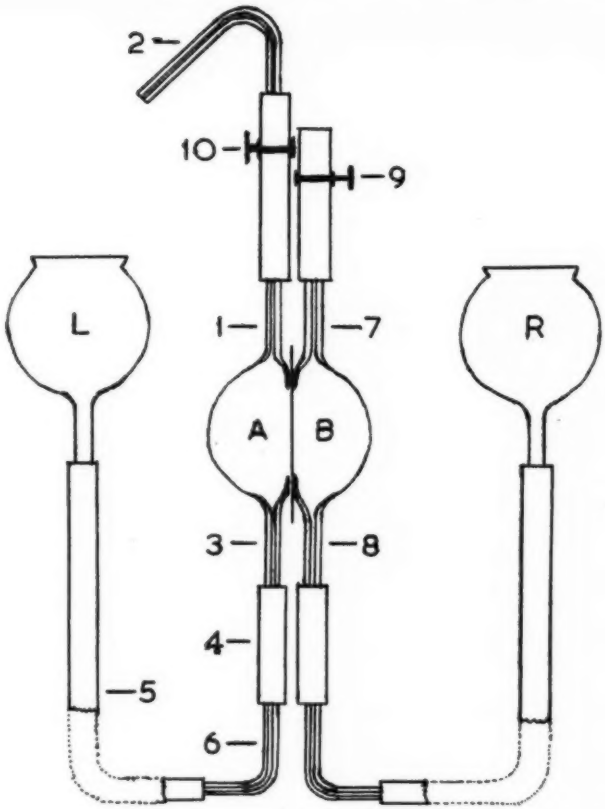


FIG. 2.

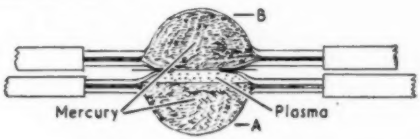


FIG. 3.

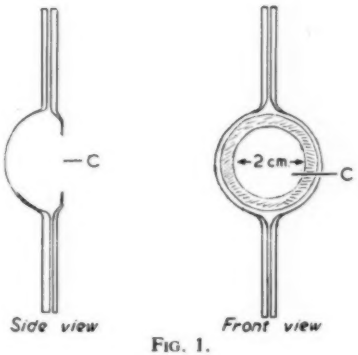


FIG. 1.

(2). The other limb (3) was connected by pressure tube (4 and 5) and an angle piece capillary tube (6) to a glass funnel (L). One limb (7) of half-cell (3) was closed by a pressure tube and screw clip (9). Limb (8) was connected in the same way as (3) to a glass funnel (R). Both systems, including the bent capillary tube, were completely filled with mercury through the funnels so that no air was trapped. Both screw clips were tightened. A funnel (L) was then lowered about 10 cm. below the level of the half-cells. Plasma was then sucked into half-cell A through the capillary tube (2) by loosening the screw clip, no air being allowed to enter. The screw-clip was then closed and funnel (L) lifted about 10 cm. over the half-cell level. The half-cells were then turned through 90° to the position shown in Fig. 3, so that cell (A) should be at the bottom and the plasma should be completely sealed by mercury. The funnel was then raised 35 cm. above the plasma level. To remove the filtrate the half-cells were turned back to the position shown in Fig. 2. Funnel (L) was then lowered to 10 cm. above the half-cell level, a pipette placed into the pressure tube of short length (B), the screw clip opened, and funnel (R) carefully raised in order that all filtrate should enter the pipette. The mercury level in (R) was 2–3 cm. above plasma level during the filtration.

To obtain the optimal conditions for the ultrafiltration of the urates, we chose, after several trials, cellophane membranes "300" (moisture proof). This type of cellophane yielded a protein-free ultrafiltrate, but, because of its large pore size, afforded the minimum resistance to the filtration of the urates. The cellophane was soaked in distilled water at 0° C. for 24 hours.

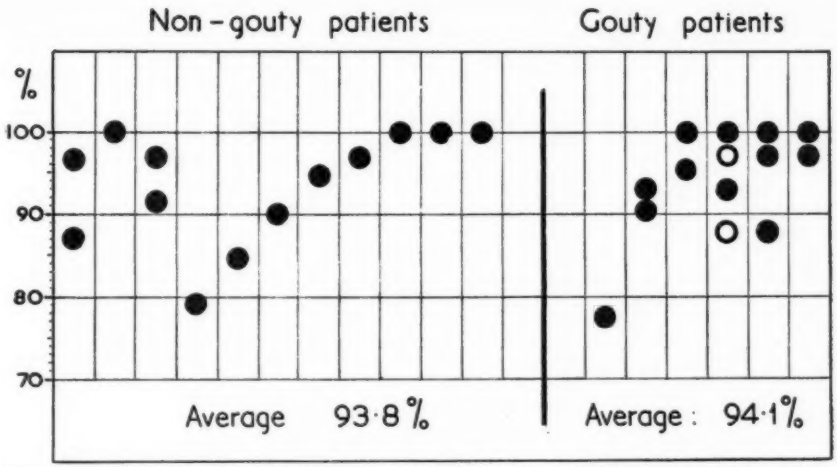
The filtration occupied 17 hours or more. Although we obtained in two instances an ultrafiltrability of 100% even after five hours, we came to the conclusion that usually a longer time is required to obtain the maximum results.

The plasma uric acid and uric acid in the ultrafiltrate were estimated by the method of Benedict as modified by Bidmead (see page 370). The determination was carried out simultaneously in both plasma and ultrafiltrate. The plasma was kept during the ultrafiltration under the same conditions as the ultrafiltrate.

Our results are presented in Fig. 4. We carried out 13 filtrations in 11 non-gouty patients, and 14 in six patients with classical gout. The plasma uric acid in the non-gouty group varied between 3.1 and 6.3 mg.% (average 4.3 mg.%), and in the gouty group from 3.5 to 8.7 mg.% (average 6.4 mg.%). The average ultrafiltrability for the non-gouty group was 93% and for the gouty ones was 94%. In both groups, with three exceptions, all the results were between 90 and 100%.

Discussion

The method of ultrafiltration employed was a modification of that used by Wolfson *et al.*, but '300" moisture-proof cellophane was used instead of "600," as it was considered that, in spite of the conditions being anaerobic, the more quickly the filtration was carried out the less chance there was of change in equilibrium in the solution. In spite of this, the time of ultrafiltration was considerable—17 hours—and the percentage of uric acid that was ultrafiltrable was on an average as high as 93% (in eight samples 100%). There was no change between the percentage ultrafiltrable from gouty and non-gouty patients. It was felt that the filtrable fraction was so large that no conclusion about the state of the urates in the blood could be drawn from this experiment. The method was considered of no value in differentiating between gouty and non-gouty individuals, contrary to previous claims.



No. of patients = 11. No. of filtrations = 13. P.U.A.: maximum = 6.1 mg.%, minimum = 3.1 mg.%, average = 4.3 mg.%.

No. of patients = 6. No. of filtrations = 14. P.U.A.: maximum = 8.7 mg.%, minimum = 3.5 mg.% (after sod. sol.), average = 6.4 mg.%. O denotes acute gouty attack.

FIG. 4.

Summary

An attempt has been made to see whether the ratio of plasma uric acid to ultrafiltrable uric acid might throw any light on the cause of undue retention or deposition of uric acid in gout.

A satisfactory method of ultrafiltration was finally found, but both in gouty and non-gouty individuals the ultrafiltrable fraction averaged between 93 and 94%. Ultrafiltration, therefore, appears of no value in differentiating patients suffering from gout from normal individuals.

We should like to thank Professor Garner for the assistance of his workshop in preparing apparatus, and Messrs. Colodense, Ltd., Bristol, for the generous supply of cellophane membranes.

We are also indebted to Dr. Pearson and Mr. Bidmead for assistance with some of the estimations, and to the Empire Rheumatism Council for some financial help.

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A MODIFIED TECHNIQUE FOR DETERMINING URIC ACID IN BLOOD AND URINE

BY

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The methods most commonly used for the determination of uric acid are those of Benedict, Behre, and Folin described in almost all textbooks of clinical biochemical technique. The Folin method has been in use in this laboratory for some time, where uric acid determinations are often carried out three or four times a day on the same patient, but it was realized that this method was not sufficiently reliable to enable such repeated determinations to be carried out with accuracy. It was found that, using the same batch of reagents, the colour intensity produced varied from day to day, and even from one set of determinations to another, so that the preparation of a standard curve was impossible. At least two standard determinations had to be carried out in parallel with the tests in order to cover the usual working range of 2–10 mg.% of uric acid (in blood plasma). One of the chief causes of error seemed to be concerned with the boiling time, and it was therefore decided to find a method which would eliminate the necessity for boiling and be sufficiently constant in its results to enable us to prepare a standard curve.

In a personal communication Dr. Wolfson, of the Michael Rees Hospital, Chicago, U.S.A., indicated a method for determining uric acid as used in his laboratory, and the modified technique outlined below is based upon this method.

Principle of the Method

The proteins are precipitated from plasma or serum using Folin's tungstic acid method. The filtrate is treated with urea-cyanide and an arseno-phosphotungstate reagent at room temperature, when a blue colour develops. After a standard period of time the colour intensity is read in a photo-electric absorptiometer and the results compared with a standard curve prepared from uric acid solutions of known concentration.

Reagents.—The following reagents were used:

(1) *0.67N Sulphuric Acid.*

(2) *10% W/V Aqueous Sodium Tungstate (A.R.).*

(3) *Urea-cyanide Reagent.*—This is prepared by dissolving 25 g. of pure sodium cyanide in 400 ml. of distilled water and adding 75 g. of urea. The volume is made up to 500 ml. with distilled water. This solution must be discarded after two months.

The urea-cyanide solution is usually slightly cloudy, due to the presence of impurities in the urea. This cloudiness does *not* interfere with the final colour reaction and may well be ignored. Some workers advise shaking the solution with 3–5 g. of calcium

oxide, allowing to settle, and filtering. The clear filtrate is said to produce 15–30% more colour, but in my experience a water-clear filtrate was obtained which interfered with the final colour reaction, either producing a precipitate, or changing the colour to a greenish tint, depending upon the sample of calcium oxide used. This is due to the presence of excess calcium hydroxide in the urea-cyanide solution. Hawk and Bergeim (1931) advise allowing the solution to stand for a week or more, when the calcium hydroxide will slowly form carbonate and be precipitated. The solution is then filtered once again. Alternatively the precipitate in the final colour reaction, due to the formation of calcium phosphate with the Benedict's reagent, can be centrifuged, and the supernatant used for the estimation of the colour. Either method is somewhat tedious, and it is not considered that any worthwhile advantage is gained by using the calcium oxide. The urea-cyanide solution is therefore used without attempting to clarify it.

(4) *Arseno-phosphotungstate Reagent* (*Benedict*).—This reagent is made up by dissolving 50 g. of molybdate-free sodium tungstate in 300 ml. of distilled water, followed by 25 g. of arsenic pentoxide. When solution has been accomplished, 12.5 ml. of syrupy phosphoric acid and 10 ml. of concentrated hydrochloric acid are added. The solution is boiled for 20 minutes, cooled, and made up to 500 ml. with distilled water in a volumetric flask. This solution keeps indefinitely. *Do not pipette. This solution is poisonous.*

(5) *Stock Solution of Uric Acid.*—This is Folin's solution prepared as follows: Lithium carbonate, 0.6 g., is weighed and dissolved in 150 ml. of distilled water. The solution is heated to 60° C. Pure uric acid, 1 g., is weighed accurately and transferred to a 1-litre volumetric flask, and the warm lithium carbonate solution is then added to the uric acid and shaken until the uric acid has dissolved (about five minutes). The solution is cooled under the cold-water tap and 20 ml. of 40% formalin and 10 ml. of 50% v/v acetic acid added. Finally the solution is made up to volume with distilled water and transferred to a dark-glass bottle. This solution keeps indefinitely if stored away from the light, and contains 1 mg. of uric acid in 1 ml.

Method

Plasma or Serum.—Care should be taken to avoid haemolysis when collecting samples of blood for plasma or serum uric acid determination. In a centrifuge tube are placed 7 ml. of distilled water, 1 ml. of plasma, and 1 ml. of 0.67N sulphuric acid followed by 1 ml. of 10% sodium tungstate solution are added. The solution is mixed by inversion and centrifuged. The supernatant liquid should be water-clear.

In a clean centrifuge tube, graduated to 10 ml., are placed 5 ml. of filtrate, 2 ml. of urea-cyanide solution, 0.2 ml. of arseno-phosphotungstate reagent, and made up to 10 ml. with distilled water, mixed immediately, and stood for five minutes at room temperature. It is read in the photo-electric absorptiometer using a dark-red filter (Ilford 608).

When a number of tests are being carried out at the same time the five minutes should be timed from the mixing of the *first* tube. If the colour is too deep, so that the reading is off the scale, repeat the test using 2.5 ml. of protein-free filtrate and double the result obtained from the chart.

A blank test is prepared using 5 ml. of distilled water, 2 ml. of urea-cyanide solution, and 0.2 ml. of reagent. This blank should be clear and colourless to the naked eye.

Urine.—Whenever urine is collected it should be kept frozen. If this is not possible, thymol or other preservative must be added. On removing from the freezing chamber the urine is warmed to 50° C. and shaken well.

Urine, 1 ml., is placed in a 100-ml. graduated flask and made up to volume with distilled water. Then 5 ml. of diluted urine is taken, and the procedure is as for plasma filtrate above. It must be emphasized that once the urine has been diluted the test must be carried out without delay, since the uric acid content of this dilute solution deteriorates rapidly after standing for about an hour.

Preparation of Standard Curve

The uric acid stock solution is suitably diluted with distilled water to give a set of standard solutions covering the desired working range. For example, 1 ml. of stock solution is diluted to 100 ml. with distilled water. Of this, 5 ml. used for the test represents a concentration of 10 mg. per 100 ml. of plasma serum, etc.

4	ml.	represents	8	mg.	per	100	ml.	of	plasma, serum, etc.
3	,,	,,	6	,,	,,	100	,,	,,	,, ,,
2	,,	,,	4	,,	,,	100	,,	,,	,, ,,
1	,,	,,	2	,,	,,	100	,,	,,	,, ,,

These volumes are placed in test-tubes graduated to 10 ml., made up where necessary to 5 ml. with distilled water, the urea-cyanide and reagent added as before, and the results plotted on a graph to give the standard curve. This should be carried out at least three times, preferably on different days, and the readings should not vary by more than one degree on the absorptiometer scale.

The standard solutions must be used immediately after preparation as they are not stable, and the pipetting of these standards must be carried out as accurately as possible, otherwise duplicate results will not be obtained.

It is important that the temperature at which the colour reaction takes place should be fixed within fairly narrow limits. For example, if the standard curve is prepared at a temperature of 20° C., then all subsequent tests must be carried out at this temperature. A fall of 3–5° C. in the laboratory temperature slows up the colour production and produces low results. Whenever a fresh reagent is made up (urea-cyanide or arseno-phosphotungstate) the standard curve should be checked to make sure that no significant deviation from it has occurred. So far, using the same brand of reagents, no deviation from the original curve has occurred during the past three months.

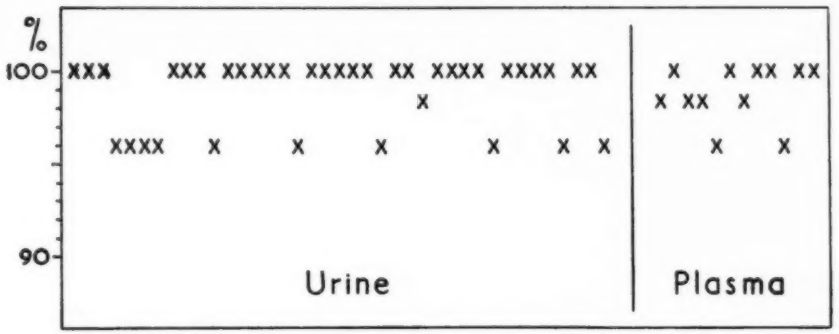
Accuracy of the Method

A number of tests were carried out to determine the percentage of uric acid recovered after the addition of a known amount of pure uric acid to the sample. In a series of 50 tests, including both plasma and urine, the uric acid content was determined in the usual way, and to a duplicate sample a known amount of uric acid stock solution was added. The uric acid content of each was determined at the same time. It was found that the amount of uric acid added was quantitatively recovered in the majority of samples thus treated. The following is a summary of the results in 50 tests.

Number	in	which	100%	recovery	was	obtained	=	35	(70%	of	total)
,,	,,	,,	98%	,,	,,	,,	=	5	(10%	,,	,,)
,,	,,	,,	96%	,,	,,	,,	=	10	(20%	,,	,,)

Fig. 1 shows the percentage recovery for plasma and urine.

The advantages of this technique lie in its simplicity, accuracy, and speed of operation. It is quicker than the Folin method, and a large number of tests can be



handled easily in a comparatively small space, since the reaction is carried out in normal size test-tubes. This is a distinct advantage over the older methods where large boiling tubes must be employed. The fact that it gives constantly reproducible results, enabling a standard curve to be prepared, is the major consideration in adopting this technique.

Summary

A modified technique for the determination of uric acid is discussed. The advantages of the method are its simplicity, economy of time and reagents, and constancy of results. Details are given of the accuracy of the method in recovering known amounts of uric acid.

Acknowledgments are due to Dr. G. D. Kersley, Director of the Rheumatism Research Unit of the South-West and Oxford Regions, for permission to publish this paper, and to Dr. E. Bene for his help and suggestions in its preparation.

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J. clin. Path. (1951), **4**, 374.

THE DISC TECHNIQUE FOR DETERMINING SENSITIVITY TO THE ANTIBIOTICS

BY

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(RECEIVED FOR PUBLICATION APRIL 16, 1951)

The range of antibiotics available for therapy is extending steadily, and in consequence there is an increasing demand on the services of the laboratory. Chloramphenicol has now been added to the free list, aureomycin is being used for certain prescribed conditions, and terramycin is generally available in the U.S.A. This form of therapy should be subjected to bacteriological control, and it is therefore important to have a simple and reliable method of determining sensitivity.

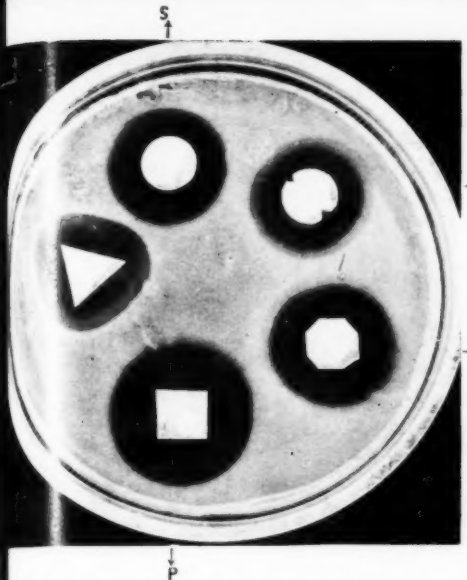
Various methods are used for these tests, and the serial dilution and the disc techniques are probably the most popular. The serial dilution method is reliable, but it is time-consuming and consequently is of limited value as a routine test.

The disc technique is now widely used for routine purposes, as a quick result can be obtained. This is a simple adaptation of the original cylinder method, in which, it should be appreciated, the assays were made at least in duplicate under carefully controlled conditions. The demand for quick results has produced a variety of disc methods, some of which have little, apart from simplicity, to offer. It should, however, be accepted that, unless a consistent standard technique is employed, the results may be very misleading. This is a matter of considerable importance if the tests are used for therapeutic control.

There are many sources of error in the disc technique, and irregular results are not uncommon. The importance of the depth of agar in the assay plates has been discussed by Hayes (1945). Some other factors are now considered.

Nature of the Disc

Two methods of using the filter-paper disc are practised, " wet " and " dry." The dried disc technique has many advantages over the wet method, in which the antibiotic may be added to the disc at the time of assay in a variety of ways, some of which are far from reliable. Dried discs can be readily prepared to a standard size and impregnated with a constant amount of antibiotic; they are easily manipulated; they can be kept for several weeks without loss of potency, but weekly control tests should be made. The test is simple and produces consistent results provided a satisfactory technique is used (Figs. 1*a* and *b*).



1a.—*Staph. pyogenes* with standard discs (on agar). Incubated for 24 hours at 37° C.; note development of secondary growth around A disc.

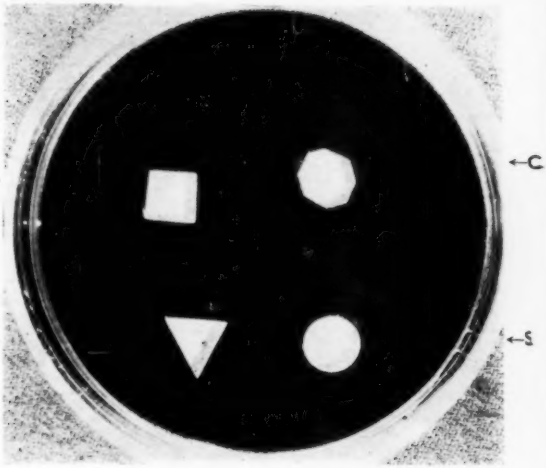


FIG. 1*b*.—*Strept. haemolyticus* (Group A) on blood-agar with standard discs.

P = penicillin (1.5 units), A = aureomycin (10 μg.), S = streptomycin (33 μg.), T = terramycin (10 μg.), and C = chloramphenicol (33 μg.).

Strength of the Antibiotic

It is important that the amount of antibiotic used in the tests should be adjusted so that zones of inhibition are given only by organisms whose sensitivity is within therapeutic limits. Thompson (1950) used dried filter-paper discs (Postlip Mills No. 633 or Green's No. 401) and recommended concentrations of (per ml.) penicillin, 1,000 units, streptomycin, 10,000 μg., aureomycin, 2,500 μg., and chloramphenicol (chloromycetin), 2,500 μg.; one drop (0.02 ml.) was added to each disc. He claimed that the zones gave a fairly accurate guide to the sensitivity of the organisms and to the adequacy of the antibiotic dosage. These concentrations are, however, greater than those generally used in the wet disc technique.

Tests have been carried out to determine the effect on the final result of varying the concentration of antibiotic on the discs. As the amount of antibiotics added to each disc is carefully controlled, it appeared an advantage to indicate the actual amount of antibiotic on the individual discs rather than the strength per ml. of the initial solutions. The quantity added to the discs varies in different laboratories; in some a drop is added by means of a pipette or platinum loop, in others the discs are soaked in the antibiotic solutions. Therefore, while the strength of the original solutions might be the same, the final concentrations on the discs might vary considerably.

Standard discs (Whatman No. 1 or Postlip Mills No. 633 filter-paper) were sterilized in the hot air oven, impregnated with varying strengths of the antibiotics by the addition of a standard drop, and then dried in the incubator. Different shapes were used for the various antibiotics, but the size was approximately the same. The following amounts per disc were found to give results of practical value: penicillin 1.5 unit, streptomycin 33 μg.,

aureomycin 10 μg., terramycin 10 μg., and chloramphenicol (chloromycetin) 33 μg. These were prepared by adding to each disc one drop (0.033 ml.) of the following strengths: penicillin 45 u./ml., streptomycin 1,000 μg./ml., aureomycin 300 μg./ml., terramycin 300 μg., and chloromycetin 1,000 μg./ml. (Fig. 1).*

There was an appreciable margin of error; a twofold increase or decrease did not significantly affect the results, but the use of quantities greatly in excess of these values tended to produce zones of inhibition around relatively insensitive organisms (Fig. 2). Thus organisms which are relatively resistant may appear to be quite sensitive by these tests if an excessive concentration of antibiotics is used. The converse also applies.

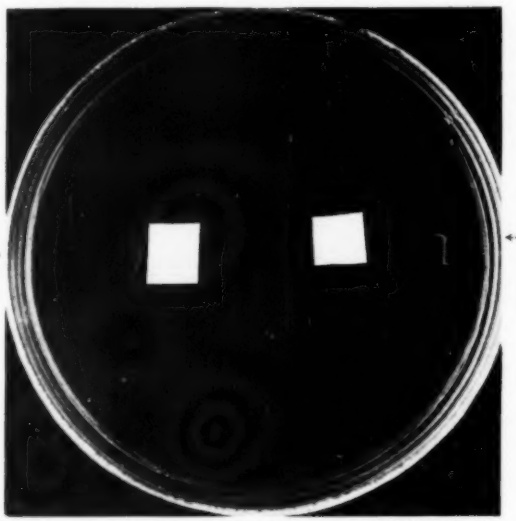


FIG. 2.—Plate seeded with *Staph. pyogenes* (inhibited by 6 u./ml. in the serial dilution test) with A concentrated disc (33 units) and B weak disc (1 unit).

Tests were made, in parallel with the serial dilution method, on a wide range of organisms. Agar or blood-agar plates were uniformly seeded, usually with a young broth culture, before the addition of the discs and finally incubated at 37° C. for 18 hours; after this time the aureomycin discs rapidly lose potency and secondary growth occurs. The results given by the dried discs were in good agreement with those obtained by the dilution technique.

Size of Inoculum

In the original "cylinder" tests the plates were seeded by flooding the medium with a broth culture and then any excess fluid was removed; in this way a uniform confluent growth was produced. It has now become routine practice to add the discs to any form of inoculum, including original material, e.g., pus, swabs, sputum, containing one or more organisms and irrespective of the number of bacteria. It has been suggested that the size of the inoculum has no appreciable effect on the zoning (Thompson, 1950). This is contrary to our experience. It has frequently been observed, particularly with resistant staphylococci (penicillinase-producers), that zones of inhibition may be obtained with a light bacterial inoculum, i.e., one giving individual colonies, while a heavy inoculum with the same organism is quite resistant. This is illustrated in Fig. 3. It is consequently important that a standard

* Blotting-paper discs (8 mm. diameter), of different colours and impregnated by means of a "50 dropper" to give the same values, are now being used.

inoculum should be used for the test. Flooding the medium with a young broth culture has given the best results, but heavy and even seeding from a solid medium with a platinum loop has also proved satisfactory.

In order to save time, and this is often an important consideration, the tests can sometimes be carried out directly on material collected from the patient. It is, however, important that the results should be confirmed by the use of the standard technique; this is particularly important if there is a mixed or light growth.

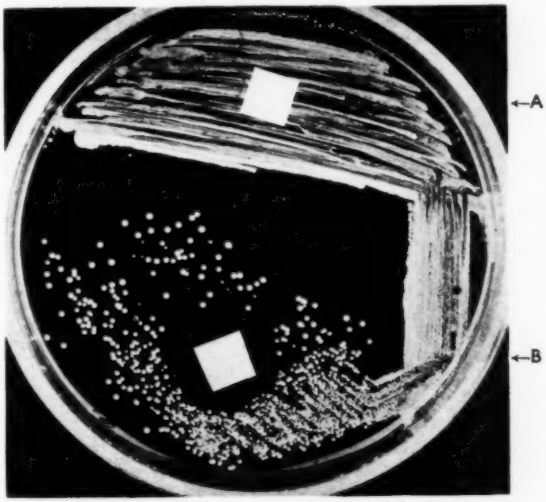


FIG. 3.—A resistant *Staph. pyogenes* (inhibited by 100 u./ml. in the tube method) with dried penicillin discs (33 units) at A heavy inoculum, no inhibition; at B light inoculum, zone of inhibition indicating sensitivity.

Summary

The dried disc method is a simple, rapid, but crude method of determining sensitivity to the antibiotics. The technique must be standardized in order to minimize the inherent errors of the test; it is important to note that a uniform heavy growth is required and that the concentration of the antibiotic should be carefully controlled. Provided these requirements are fulfilled, valuable practical information of the sensitivity of an organism can be rapidly obtained. In any doubtful case, more accurate methods of assay should be carried out.

We wish to thank the Department of Clinical Photography, Manchester Royal Infirmary, for the figures, Messrs. Cyanamid Products, Ltd., for the supply of standard aureomycin hydrochloride, Messrs. Parke, Davis & Co. for the pure chloromycetin, and the M.R.C. for the terramycin.

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J. clin. Path. (1951), **4**, 378.

AN INSTRUMENT FOR COMBINED STERNAL BIOPSY AND ASPIRATION

BY

L. BERNSTOCK AND H. STERNDALE

From the Department of Pathology, Hackney Hospital, London

(RECEIVED FOR PUBLICATION MARCH 8, 1951)

It is now universally agreed that examination of bone marrow aspiration material is essential for unravelling many haematological problems. In some cases, however, it is extremely difficult, or even impossible, to obtain specimens, as for example in the myelosclerotic and refractory anaemias. In the former, examination of the bone itself would be of value, and in the latter one would like to see the arrangement of the marrow elements *in situ*. In the past these difficulties were overcome by subjecting the patient to a minor operation when a bone biopsy was taken.

With the instrument devised by Dameshek (1935), although satisfactory, a small skin incision before taking the biopsy specimen was found essential.

In 1943 Turkel and Bethell produced an instrument which performed a sternal biopsy without the disadvantage of a skin incision and subsequent scarring. The instrument was simple to use and produced a thin core of bone with marrow attached. We have used this needle on several occasions, but found, as did the original authors, that if both biopsy and aspiration material were required a second needle of the usual Sahla pattern had to be inserted as a second operation. Our present modification of this needle, we think, overcomes this disadvantage and now affords a simple method of obtaining both aspiration and biopsy specimens for examination in the one puncture.

The Apparatus

The apparatus consists of two needles, an outer guiding and aspirating needle, and an inner trephine. The outer needle is built on the general pattern of the Sahla type with an adjustable guard 9 cm. long and a bore of 14 British wire gauge. At one end it has a bevelled cutting end, and is fitted with a stylet. The upper portion of the needle has a fitting to which a syringe with a Luer mounting can be attached.

The inner needle consists of a trephine 11.15 cm. long and a bore of 17 British wire gauge. A metal shoulder, to which are attached metal flanges for ease of handling, is at the upper end. The trephine slips easily into the outer component of the apparatus, extending 0.8 cm., the maximum distance being fixed by the impingement of the metal shoulder on the outer needle head. Its upper portion has a fitting to which a syringe of record pattern can be attached. A long stylet is provided with the trephine which, when in position, extends 0.9 cm. beyond the tip. The sole purpose of the stylet is to help to eject the biopsy.

Method

The site selected for this procedure is that for routine sternal marrow aspiration. We normally take a point opposite the second or third ribs, slightly to the left of the mid-line.

Skin, subcutaneous tissue, and periosteum are infiltrated around this point with 2% procaine.

The outer needle, with stylet in position, is now inserted through the skin. When periosteum is reached the stylet is removed and the needle is adjusted so that it is lying at 75° with the sternum. The position is maintained with the left hand: The trephine, without its stylet, is now introduced within the outer needle until it reaches periosteum. By a circular boring motion and using only moderate pressure the trephine is sunk up to the metal shoulder. At this point a final twist will finally detach the biopsy from the surrounding structures.

The trephine is removed and handed to an assistant, who, with the aid of the stylet, can express the biopsy into the fixative, which in our cases has been Zenkers fluid.

The stylet of the outer needle is now reintroduced and the procedure is as for a routine aspiration.

Before making films excess blood should be removed from the marrow particles.

The whole of the procedure takes little longer than the accustomed marrow aspiration, causes the patient no greater discomfort, and in many cases affords greater assistance in diagnosis.

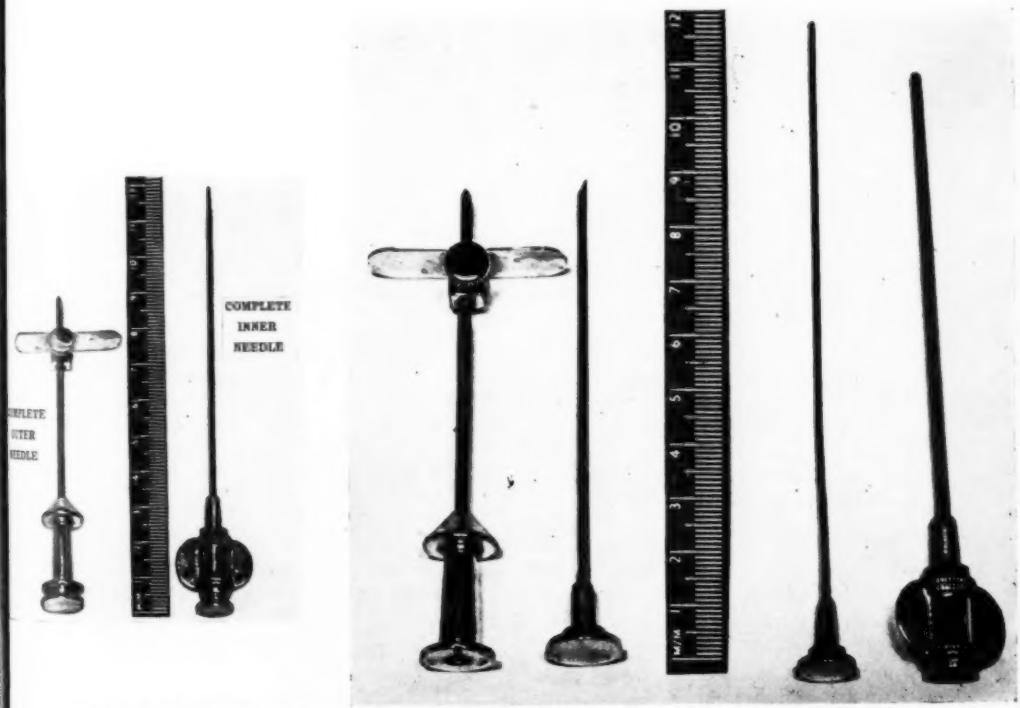

FIG. 1 and 2.—Outer needle and trephine with stylets *in situ*, and component parts of instrument.

Our thanks are due to Dr. L. Steingold for his help with the preparation of the histological specimens; to Dr. H. B. May for his helpful criticism; to the Nucleus Photographic Specialists for the photographs; and to Charles Thackray for making the instrument to our specification.

The instrument is now available from Charles Thackray, Ltd., 38, Welbeck Street, London, W.1.

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J. clin. Path. (1951), 4, 381.

EVALUATION OF A METHOD OF ESTIMATING THE BASAL METABOLIC RATE

BY

J. G. ALEXANDER

From the Department of Pathology, Liverpool Royal Infirmary

(RECEIVED FOR PUBLICATION JULY 11, 1949)

In 1946 Barman and Porcile published a method whereby an accurate " oxygen line " could be drawn by making the patient expire maximally at intervals during basal metabolism estimation with the Benedict Roth apparatus. They only claimed that it was of use in cases with an irregular respiratory rhythm.

The Barman and Porcile method is as follows.

While the patient is breathing normally into the spirometer he is instructed to make a deep expiration. It is important to ensure the maximal possible emptying of the lung so that all the reserve (supplemental) air will be expelled into the spirometer. To achieve this the subject must be stimulated to go on expelling air until the expiratory line flattens out. This process is repeated once or twice at two to six minute intervals. The oxygen line is traced by joining the points of extreme expiration.

Since March, 1948, this method has been used with an alcohol-tested Benedict Roth apparatus at the Liverpool Royal Infirmary, and was tried in 29 out of 151 estimations (18%) of basal metabolism with irregular breathing. The method was also used in several other estimations, in some of which I was the subject.

The first step was to evaluate the method when used on normal cooperative, intelligent persons. This was done in 10 cases including myself. The procedure adopted was to determine the basal metabolic rate using the usual method first and then the maximal expiration method. It was found that the oxygen consumption per minute was higher with the maximal expiration method in six out of the 10 cases. It was also found that the points at the end of each maximal expiration did not always form a straight line. In spite of these results it was felt that the drawing of an accurate " oxygen line " in some cases of irregular respiratory rhythm was so difficult that it was worth trying the maximal expiration method. It was soon discovered also that some patients were so unintelligent that they did not seem to be able to understand the simple instruction " breathe out as much as you can." Furthermore, some very nervous patients were confused by these instructions, and the oxygen consumption rose quite considerably after they had tried to carry them out.

Of the 17 patients on whom it was found possible to use the method, eight gave oxygen consumption results by the maximal expiration method which were, on an average, 20 ml. per minute lower than those obtained by drawing a line as best one could through the points of normal expiration. In the other seven patients the maximal expiration results were higher.

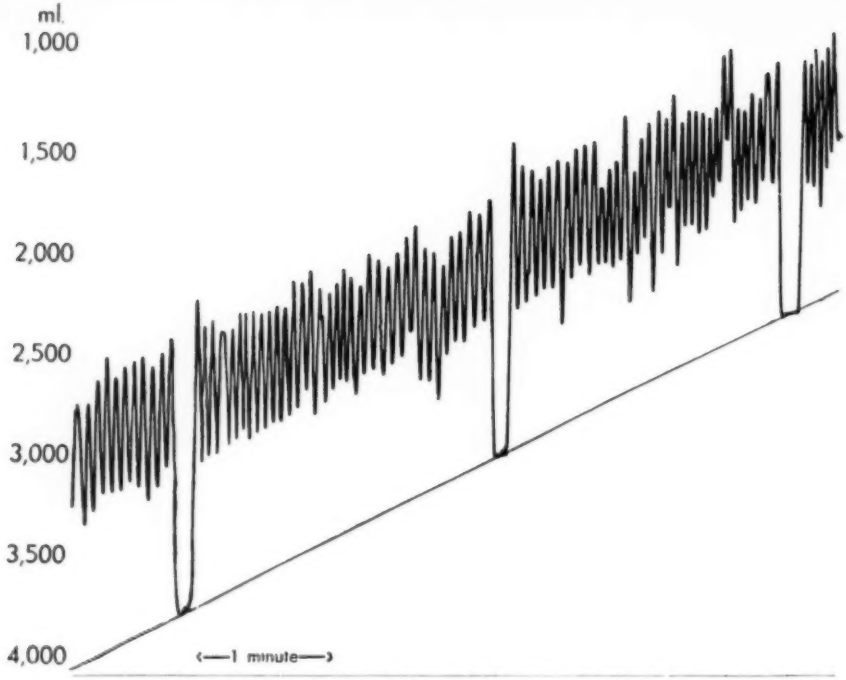

FIG. 1.—Specimen record in case of irregular respiration.

Summary

The method has been found to give inconsistent results, but may be helpful in a few isolated cases. It is emphasized that the patient should be intelligent enough to carry out the procedure.

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Barman, J. M., and Porcile, E. (1946). *J. Lab. clin. Med.*, **31**, 1254.

J. clin. Path. (1951), **4**, 383.

A CELL-SUSPENSION MIXER

BY

G. A. MATTHEWS

From the West Herts Hospital, Hemel Hempstead

(RECEIVED FOR PUBLICATION APRIL 11, 1951)

For the fundamental idea of mixing cell suspensions by rotation at a low speed I am indebted to Dr. J. V. Dacie's book *Practical Haematology* (1950).

The machine depicted here carries a variety of spring clips, to take different sized containers, on a turntable inclined at an angle of 45°. The head rotates slowly so that the air bubbles in the containers travel completely up and down with each revolution. The mixer is electrically driven and is silent in operation. There is an on-off switch, but the rotation can be stopped briefly by hand without using this if desired.

The following advantages may be claimed for the use of this machine in haematology. Red and white cell suspensions can be made with 20 ml. and 50 ml. pipettes, thus dispensing with the less accurate bulb pipettes and reducing the error due to dilution. The tubes are then clipped to the mixer and after a lapse of three minutes can be removed as and when required for charging counting chambers with Pasteur pipettes. If Wintrobe's method of doing blood counts is used the mixing of the initial oxalated blood sample is done by the machine as well. The setting up of a number of sedimentation tubes is greatly facilitated.

The machine can be used to defibrinate blood given a container with a snare attached to the cap to collect the fibrin.

It has been found that very even mixing of cell-suspensions is achieved, with a saving of time proportionate to the number of blood counts undertaken.

The machine can be obtained from Messrs. Hawkesley & Sons, Ltd., 17, New Cavendish Street, London, W.1.

J. clin. Path. (1951), **4**, 384.

ABSTRACTS

This section of the JOURNAL *is published in collaboration with the two abstracting journals, Abstracts of World Medicine, and Abstracts of World Surgery, Obstetrics and Gynaecology, published by the British Medical Association. In this* JOURNAL *some of the more important articles on subjects of interest to clinical pathologists are selected for abstract, and these are classified into four sections: bacteriology; biochemistry; haematology; and morbid anatomy and histology.*

BACTERIOLOGY

Synergism and Antagonism as Displayed by Certain Antibacterial Substances. BIGGER, J. W. (1950). *Lancet*, **2**, 46.

This paper records the results of an investigation undertaken to determine the interaction of sulphathiazole, penicillin, streptomycin, chloramphenicol, boric acid, and *p*-aminosalicylic acid when tested *in vitro* in pairs against *Bacterium coli*. A synthetic medium was used and its *p*H adjusted to 7.5. The results are summarized in the following table:

	Sulphathiazole	Penicillin	Streptomycin	Chloramphenicol	Boric Acid	*p*-Aminosalicylic Acid
Sulphathiazole	—	S	S	AS	A	AS
Penicillin ..	S	—	S	S	AS	S
Streptomycin	S	S	—	S	S	AS
Chloramphenicol	S	S	S	—	S	S
Boric acid ..	A	S	S	S	—	S
p-Aminosalicylic acid	S	S	S	S	S	—

To be read downwards, not across. S = Synergism. A = Antagonism.

Antagonism was demonstrated in five combinations, but only in the case of boric acid and sulphathiazole was each constituent antagonistic to the other. The only other marked and permanent antagonisms were those of *p*-aminosalicylic acid to sulphathiazole and of boric acid to penicillin. In the case of some combinations both antagonism and synergism were found at different concentrations of the antibiotics. In two cases of antagonism (chloramphenicol to sulphathiazole and *p*-aminosalicylic acid to streptomycin) the antagonism which was obvious for the first day or two was replaced by synergism within 3 days.

The theoretical and practical importance of these findings is discussed. The author's general conclusion is that, " as regards the six substances investigated, the synergism which, with a single exception, occurs between them might be of considerable value and the antagonism which has been demonstrated in a few cases is very unlikely ever to represent a real danger." *A. W. H. Foxell.*

Use of Penicillin O in Patients Hypersensitive to Penicillin G. VOLINI, I. F., SHALES, W. H., and FELSENFELD, O. (1950). *J. Amer. med. Ass.*, **143**, 794.

Several workers have reported that persons hypersensitive to penicillin G may show no reaction to some of the biosynthetic penicillins. One of these is allylmercaptomethyl penicillin, or penicillin O. The minimum inhibitory concentrations of penicillin O for Gram-positive cocci are the same as, or double, those of penicillin G. Furthermore, penicillin O was found to be less toxic and less irritating than penicillin G when injected subcutaneously into mice. The absorption and excretion curves of these two penicillins are essentially the same.

Penicillin O was administered to 57 patients who had clinical symptoms of hypersensitivity to penicillin G within a preceding period of not more than 200 days. Twenty of them had upper respiratory infections, and were given inhalations of penicillin-O dust, 100,000 units every 4 hours for 7 days.

All recovered without complications and showed no allergic phenomena. In 23 cases of tonsillitis and pharyngitis due to β-haemolytic streptococci the patient received 10 troches containing 3,000 units of penicillin O daily for 3 days. The results were excellent in 15, good in 5, and poor in 3 cases. Fourteen patients received courses of injections of penicillin O, following which penicillin G was given and was well tolerated. It appears that penicillin O had a desensitizing effect. Seven of the cases are reported in detail and a further 7 in tabular form. In every case penicillin O was as effective as penicillin G. *A. W. H. Foxell.*

Prolongation of Therapeutic Levels of Penicillin in Blood by Implantation of Procaine-Penicillin Tablets. HUIDOBRO, F., CROXATTO, R., and LUCCHINI, A. (1950). *Rev. med. Chile*, **78**, 363.

Subcutaneous or intramuscular implantation of procaine-penicillin tablets prolongs therapeutic blood levels for several days. Tablets consisted of procaine penicillin G with 20% ox fat. Implantation of 400- to 800-mg. tablets gave a detectable level of penicillin in the blood for 14 days, and of a 1-g. tablet for 23 days. The tablets caused no local or general reactions, and the authors discuss their possible uses, especially in preventing post-operative infection and in the treatment of syphilis. *René Méndez.*

Blood Levels and Urinary Excretion of Aureomycin after Intravenous and Intramuscular Administration. GOCKE, T. M., WELLS, E. B., COLLINS, H. S., and FINLAND, M. (1950). *J. Lab. clin. Med.*, **36**, 100.

Previous reports on the absorption and excretion of aureomycin are reviewed and the authors' own investigations described. The concentration of aureomycin in the blood and urine following the administration of 100- and 500-mg. doses intravenously and intramuscularly was determined in groups of 2 or 3 normal subjects, or patients convalescent from minor illnesses who had not been treated with any antibacterial agent. All had normal renal function, and the normal undiluted urine had no antibacterial action. For intravenous injection crystalline aureomycin hydrochloride was dissolved in 5% aqueous solution of dextrose; solutions for intramuscular injection contained leucine buffer and procaine in addition. Serum and urine concentrations of aureomycin were determined by a modification of the Rammelkamp method for penicillin.

On intravenous administration by continuous drip infusion over ½ to 1 hour, a decreasing concentration of aureomycin was detectable in the serum for 12 hours after a 500-mg. dose and for 2 to 4 hours after a 100-mg. dose. No aureomycin could be detected in the blood after similar doses given intramuscularly. On intravenous injection the most rapid rate of excretion in the urine occurred within 8 to 12 hours after a 500-mg. dose, but when the same dose, which was highly irritating, was given intramuscularly it was excreted irregularly. A 100-mg. dose given intramuscularly was excreted rapidly during the first 3 hours. Appreciable amounts of aureomycin were excreted for 2 to 4 days after intravenous injection, and for 8 days after intramuscular injection. After intravenous injection 26 to 50% of the dose was recovered from the urine as active aureomycin, whereas 13 to 24% was recovered after intramuscular injection. In view of the rapid deterioration of aureomycin in neutral or alkaline solution, and particularly in view of reports that aureomycin is protected from deterioration in tissues, it is concluded that much of the loss of antibiotic occurred during manipulation of the samples. The marked irritation caused by intramuscular injection of aureomycin tends to keep the agent localized at the site of injection, and the intravenous route is therefore preferable. Rough estimations indicate that aureomycin is cleared from the plasma at the rate of 40 to 70 ml. of plasma per minute, which is similar to the clearance rate for streptomycin. It is concluded that aureomycin is excreted by glomerular filtration alone.

P. B. Marshall.

Clinical Observations on Terramycin. BLAKE, F. G., FRIOU, G. J., and WAGNER, R. R. (1950). *Yale J. Biol. Med.*, **28**, 495.

This paper records the results of treatment with terramycin of 15 patients with various infections. Terramycin hydrochloride was given orally in all cases. Various dosage schemes were employed, but treatment usually consisted of an initial dose of 1 g. repeated 4-hourly for 3 doses, followed by 3 to 6 g. a day at 3- or 4-hourly intervals. Among the conditions in which good results were obtained were pneumococcal lobar pneumonia and infections due to staphylococci, *Streptococcus viridans*, *Strep. faecalis*, some strains of *Bacterium coli*, *Bacillus mucosus capsulatus*, *Proteus vulgaris*, and Vincent's organisms. In 2 cases of subacute bacterial endocarditis the immediate response was good, but relapse occurred as soon as treatment was stopped. The only toxic effects encountered were nausea, vomiting, and diarrhoea, which occurred in 12 of the 15 cases, but were troublesome in only 3. *A. W. H. Foxell.*

Clinical Observations in an Epidemic of Harvest Fever. LITZNER, S., and HAHN, H. (1950). *Dtsch. med. Wschr.*, **75**, 882.

Harvest fever, caused by one of three species of leptospira, *L. grippo-typhosa*, *L. sejroe*, and *L. australis*, is unknown in Great Britain. It is rare in western Germany, and the author of this interesting paper describes in detail the clinical features of the disease as observed in 1949 during the first epidemic to be recorded in Lower Saxony, in the district of Wolfenbüttel. In July and August, 43 patients were admitted to the municipal hospital there, most of whom had been engaged in the harvesting of peas, while in the few remaining cases contact with freshly harvested peas, or in one case with the fields from which these had been harvested, could be proved. Headache and pains in the limbs and joints were typical initial symptoms, and giddiness, nausea, and vomiting were also common. The temperature was high (between 39° and 40° C.), and usually fell by lysis over a period lasting up to 14 days; after an afebrile interval of 1 to 5 days a secondary rise was frequently observed for 1 to 4 days. Injection of the conjunctiva and sclera was common. Jaundice was not observed, but rashes of varying description and intensity were frequent. Complete recovery took 10 to 12 weeks, or even longer; no fatalities were reported. The incubation period was 9 to 16 days. Positive agglutination reactions were obtained in all cases, and leptospirae were also cultured from the blood. Coincidentally with the occurrence of this epidemic a large increase of the murine population was noticed, and leptospirae were isolated from the kidneys of over 80% of mice trapped in the area. *W. G. Harding.*

The Influence of a New Benzoic Acid Derivative on the Metabolism of *para*-Aminosalicylic Acid (PAS) and Penicillin. BOGER, W. P., BEATTY, J. O., PITTS, F. W., and FLIPPIN, H. F. (1950). *Ann. intern. Med.*, **33**, 18.

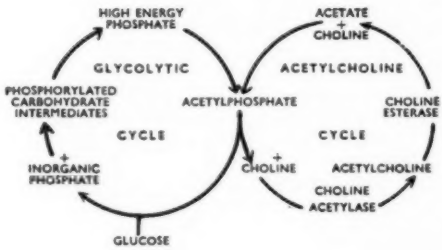
It has been found that "benemid," *p*-(di-*n*-propyl-sulphamyl)-benzoic acid, has the property of enhancing the concentrations of both penicillin and *p*-aminosalicylic acid (PAS) in plasma. Single oral doses of 1 and 2 g. were effective in doubling the plasma concentration of penicillin and PAS for periods of up to 8 hours. Most of the patients (50 were studied) received "benemid" for no longer than 2 to 3 days, but some received the compound for 40 days in an oral daily dose of 1.5 g. without toxic symptoms. *R. Wien.*

BIOCHEMISTRY

Some Problems of Potassium Metabolism. WELLER, J. M., and TAYLOR, I. M. (1950). *Ann. intern. Med.*, **33**, 607.

A theory of potassium metabolism is put forward, based on the following considerations and assumptions: (1) Potassium moves all the time between cells and the extracellular fluid. (2) Glucose is necessary for the cellular metabolism of potassium. (3) Potassium may be necessary for the cellular metabolism of glucose. (4) Acetylcholine increases the permeability of cells to ions, and may therefore aid

the cellular metabolism of potassium. (5) Potassium may enhance the formation and breakdown of acetylcholine. The possible relation between these processes is expressed by the authors in the schema below, in which glucose is the only substance which must be constantly supplied.



It is concluded from this schema that disturbances of potassium metabolism may be caused by a breakdown of either the glucose metabolism or the acetylcholine cycle. This may be responsible for the low potassium levels in the cells, often seen in untreated diabetes, and for the low serum potassium levels often seen after injections of insulin or adrenaline. Another application of this schema was sought in periodic familial paralysis, in which attacks of paralysis are associated with low potassium levels in serum and can be brought about by giving insulin and adrenaline. It is possible that myasthenia gravis may be associated with a disturbance of potassium metabolism resulting from a lack of acetylcholine.

Iron Absorption in Infections. Recognition of Iron Deficiency in Infections by Means of the Absorption Test. Pathogenesis of the Anaemia of Infection. JASINSKI, B. (1950). *Acta haematol., Basel*, **3**, 17.

An attempt has been made to determine the extent to which iron absorption is disturbed by the presence of infection and of malignant tumours. The method used was by estimation of the serum iron concentration 1, 3, and 7 hours after an oral test dose of iron, a high rise in the serum iron level being considered indicative of a rapid absorption and so of an iron deficiency. Initial experiments showed that when iron deficiency, due to blood loss, is present, iron is readily absorbed both by febrile patients and by those with malignant disease. Cases of infective anaemia have been found which showed a rapid iron absorption and in which, therefore, iron deficiency was presumed, even when no reason for blood loss was present. Clinical improvement followed oral administration of iron in such cases. In pure infective anaemia the serum iron level is unaffected by oral iron therapy. It is considered that in these cases there is adequate iron in the tissue depots of the liver, spleen, and marrow.

[The work is based on 16 serum iron curves in cases of infection and malignant neoplasms.] *C. L. Cope.*

Diabetes Mellitus and Addison's Disease: Report of Case with Normal Urinary Corticosteroids and High Insulin Requirement. WARD, E., PARKIN, T. W., and HOWARD, L. P. (1950). *Proc. Mayo Clin.*, **25**, 145.

A detailed case report is given of a patient in whom Addison's disease supervened after he had been treated for diabetes mellitus for 13 years. Usually in such cases the insulin requirement falls owing to increased sensitivity to insulin, but this patient's insulin needs did not change appreciably. In conformity with this, his urinary output of corticosteroids was normal, and it is suggested that continued formation of corticosteroids prevented the usual increase in insulin sensitivity. The clinical signs of Addison's disease were definite, the excretion of 17-ketosteroids was low, and tests showed severe impairment of the 11-oxycorticosteroid-like function of the adrenals. The possibility of suprarenal damage which did not affect the production of one group of adrenal hormones is discussed.

D. A. K. Black.

The Appearance of Vitamin B_{12} Activity in Urine after Oral and Intramuscular Administration to Man. CHOW, B. F., LANG, C. A., DAVIS, R., CONLEY, C. L., and ELLICOTT, C. E. (1950). *Bull. Johns Hopk. Hosp.*, **57**, 156.

The only measure of the absorption of vitamin B_{12} by patients with pernicious anaemia so far employed has been the haematological response. In the experiments here reported the authors made an attempt to use the vitamin-B_{12} activity of the urine, estimated by microbiological assay, as a means of measurement of absorption. Crystalline vitamin B_{12} was given intramuscularly and by mouth to normal subjects and to untreated patients suffering from pernicious anaemia. Urine specimens were collected for one day before, and for two days after, the dose and their vitamin-B_{12} activity assayed. In some of these and in subsequent experiments 10 μg. of biotin was given at the same time, because this vitamin is known to appear quantitatively in both urine and stool specimens, and its addition thus served as a check on the adequacy of collection.

Up to about 50% of vitamin B_{12} could be detected in the urine 12 hours after parenteral administration of 100 μg., there having been no appreciable vitamin-B_{12} activity detectable in the urine previously. When doses of 100 μg. or 500 μg. of vitamin B_{12} were given orally, no vitamin-B_{12} activity could be detected in the urine either of normal subjects or of those with pernicious anaemia during the next 72 hours. Addition of 400 g. of beef, chicken, or pork to the diet of 3 normal subjects for four days did not lead to the appearance of any appreciable vitamin-B_{12} activity in the urine. It had been anticipated by the authors that in normal subjects at least some vitamin-B_{12} activity would be detectable in the urine after peroral administration of large doses of the vitamin, but that patients with pernicious anaemia would not absorb it. These experiments indicate, however, that vitamin B_{12} may be absorbed poorly or not at all even by normal individuals.

[Some haematopoietic response has been claimed after peroral ingestion of the vitamin if given in very large doses. It has also been shown that administration by mouth of only 5 to 10 μg. of vitamin B_{12} together with 100 to 150 ml. of normal gastric juice, or with extract of hog's stomach or duodenum, seems to produce a haematopoietic response in patients with pernicious anaemia which is comparable to that obtained by parenteral administration of a similarly small dose of the vitamin. The intrinsic factor may thus protect the vitamin from destruction by the gastrointestinal secretions or the microorganisms of the upper small intestine, or, on the other hand, facilitate its passage through the intestinal wall.]

Z. A. Leitner.

HAEMATOLOGY

Addisonian Pernicious Anaemia. Confirmatory Evidence of a Factor Inhibiting Erythropoiesis. THOMPSON, B. R. (1950). *Clin. Sci.*, **9**, 281.

At the Royal Victoria Infirmary, Newcastle-upon-Tyne, bone marrow cells, obtained by aspiration from cases of pernicious anaemia in relapse, were cultured in media containing varying proportions of normal plasma or serum derived from normal subjects and from patients with pernicious anaemia. The method of culture used was that of Osgood and Brownlee, and erythroblasts were classified according to the method of Davidson *et al.* (*Quart. J. Med.*, 1942, **11**, 19).

Cultures in various dilutions of normal plasma ranging from 10 to 100% showed no significant difference in the degree of maturation. But in cultures made in media containing plasma from cases of pernicious anaemia the number of immature cells present increased with the concentration of plasma present, though this effect varied with different samples of plasma. Moreover, the addition of pernicious

anaemia plasma to normal plasma produced an inhibition of the maturing effect of the latter. These findings agree with those of Rusznyak *et al.* (*Hungarica Acta med.*, 1948, **1**, 1), who suggested that such an inhibitory factor was present in the serum of patients suffering from pernicious anaemia, and that the disease was not simply due to the absence of a specific maturation element. *A. T. Macqueen.*

Paroxysmal Nocturnal Hemoglobinuria. A Specific Test for the Disease Based on the Ability of Thrombin to Activate the Hemolytic Factor. CROSBY, W. H. (1950). *Blood*, **5**, 843.

The test for paroxysmal nocturnal haemoglobinuria (PNH) described here has been developed from observations previously reported in detail. It depends on the activation by thrombin of an inert plasma factor which when active becomes specifically haemolytic against erythrocytes in PNH. The test is simple and rapidly performed; it has been carried out over a hundred times in patients with PNH. In these it was invariably positive. Erythrocytes from normal subjects and from a variety of patients with haemolytic anaemia of types other than PNH always gave negative results. [Readers are referred to the original paper for details of the test.] *A. Brown.*

Study of a Case of Purpura Associated with Bone Changes and Formation of a Gel in the Serum on Cooling. LUCEY, H. C., LEIGH, E., HOCH, H., MARRACK, J. R., and JOHNS, R. G. S. (1950). *British J. exp. Path.*, **31**, 380.

A farm labourer of 57 complained of blood spots in his skin and swellings over the bones of his legs, pains in his leg bones, and occasional small haemoptyses. Later he noticed mistiness of vision, whose disappearance was followed by transient enlargement of his salivary glands. Radiographic examination showed increased density and coarse trabeculation of the whole skeleton, with marked thickening of the cortex of the long bones. The general picture was much like that in Paget's disease, but the alkaline phosphatase level in serum was only slightly raised; the alternative diagnosis was sarcoidosis. No evidence of myelomatosis could be obtained.

The patient's serum formed a gel on cooling; it passed from fluid at 27° C. through cloudiness at 25° C. and increased viscosity at 24° C. to the formation of a solid gel at 23° C.; the whole process was reversible. The active component was a substance migrating electrophoretically with the β-globulin, but it cross-reacted with anti-γ-globulin sera. It contained 0.5% cholesterol, less than 0.03% liquid phosphorus, 7% or less total lipid, and about 5.8% carbohydrates (as galactose-mannose). Its sedimentation constant was 17.2×10^{-13}, corresponding to a molecular weight between 400,000 and 1,000,000. The tyrosine: tryptophan ratio (1.63 : 1) was lower than that found in β or γ globulin, or in fibrinogen. The protein is compared with that obtained from cases previously described, and the symptoms shown by the patients are contrasted.

C. L. Oakley.

Febrile Thrombopenic Purpura with Hemolytic Anemia and Platelet Thrombosis. GOLDENBERG, P. T., THAYER, J. E., and HASTINGS, L. P. (1950). *New Engl. J. Med.*, **243**, 252.

Several cases of the syndrome of fever, progressive anaemia, mild icterus, thrombopenia, and changes in the central nervous system have now been reported in the literature. The case reported in this paper was unusual because the onset took the form of a confusional state, for which the patient was admitted to a mental hospital. The usual manifestations of haemorrhage, especially from the vagina, severe anaemia, and constant pyrexia appeared very soon. As in other cases, there was no response to transfusion and the patient died about three weeks after the onset. Post-mortem examination showed numerous thrombi in

the blood vessels of almost every organ, including the brain. These thrombi were made up of masses of platelets, and none of the usual fibrin was present. This finding also agrees with previous descriptions. The literature and theories of aetiology are fully discussed. *M. C. G. Israëls.*

Probable Transmission of Viral Hepatitis by Ultraviolet-irradiated Plasma. Report on Three Cases. ROSENTHAL, N., BASSEN, F. A., and MICHAEL, S. R. (1950). *J. Amer. med. Ass.*, **144**, 224.

Hepatitis following the Use of Irradiated Human Plasma. BARNETT, R. N., FOX, R. A., and SNAVELY, J. G. (1950). *J. Amer. med. Ass.*, **144**, 226.

Homologous Serum Jaundice Associated with Use of Irradiated Plasma. A Preliminary Report. JAMES, G., KORNS, R. F., and WRIGHT, A. W. (1950). *J. Amer. med. Ass.*, **144**, 228.

These three communications all deal with the same subject and show conclusively that exposure of plasma to ultraviolet light does not inactivate the hepatitis virus. Altogether 16 cases of hepatitis are described, two of them fatal, following the injection of irradiated plasma. There is thus no reliable method as yet for inactivating hepatitis viruses in plasma, serum, or whole blood. *G. M. Findlay.*

Fulminating Purpura with Factor-V Deficiency. Cure by Exchange Transfusion. GASSER, C., and DE MURALT, G. (1950). *Helv. paediat. Acta*, **5**, 364.

The authors describe two severe cases of purpura fulminans following scarlet fever in a 7-year-old girl and a 6-year-old boy. The clinical picture, the pathogenesis, and the treatment of purpura fulminans are discussed and the following points are emphasized:

In these two cases a severe disturbance of coagulation existed which was due to an acute insufficiency of Owren's factor V, which was combined in one case with an excess of antithrombin of the heparin-antithrombin type. The great increase in plasma cells of the reticulum and of eosinophils in bone marrow, as well as the increase in γ globulins in the blood electrophoresis—findings which, too, were made for the first time—point to an increased production of antibodies and to an allergic diathesis. The second patient could be saved when moribund by a complete blood-exchange transfusion, while repeated smaller transfusions in the first case were unsuccessful. The exchange transfusion alone assures complete removal of abnormal products and a sufficient supply of those products which were absent in the blood. Exchange transfusion appears to be the method of choice for the treatment of purpura fulminans.—[From the authors' summary.]

MORBID ANATOMY AND HISTOLOGY

The Testis, Breast, and Prostate of Men who Die of Cirrhosis of the Liver. BENNETT, H. S., BAGGENSTOSS, A. H., and BUTT, H. R. (1950). *Amer. J. clin. Path.*, **20**, 814.

This study was prompted by the observation that, in male patients with cirrhosis of the liver, gynaecomastia, testicular atrophy, and other signs of endocrine imbalance frequently develop. It is believed that endogenous oestrogen is inactivated by the healthy liver, and that this function is impaired when the organ is grossly diseased.

The necropsy findings in 50 patients with portal cirrhosis of long standing and 50 subjects with no history of, or necropsy evidence of, liver dysfunction were compared. Special attention was paid to the histological changes in testis, breast, and prostate in both groups.

The testes of all but 3 of the patients with cirrhosis showed atrophy of a mild to severe degree. Advanced atrophy was observed in half of the cases of cirrhosis, and this was associated with marked thickening and hyalinization of the seminiferous tubules in most cases. Gross atrophy was found in only 6.1% of the controls, and tubular thickening was not greatly in evidence.

The only noteworthy change in the prostate of patients with hepatic disease was a metaplasia of the columnar-cell acinar epithelium to epithelium of a transitional type. This change was observed in 7 patients with cirrhosis and in 2 controls.

The breasts of 20 patients with hepatic cirrhosis were available for study. In 7 there was marked hyperplasia, and a definite correlation between hypertrophy of the breast and atrophy of the testis was noted. Minor degrees of hyperplasia were found in 10 out of the 50 controls.

The authors conclude that the lesions complicating cirrhosis of the liver are probably caused by an excess of endogenous oestrogen. *G. B. Forbes.*

Transfusional Siderosis. WYATT, J. P., MIGHTON, H. K., and MORAGUES, V. (1950). *Amer. J. Path.*, **26**, 883. Bibliography.

Necropsy findings, histological and chemical, are described in 8 patients with refractory anaemias treated by multiple blood transfusions. In all cases the liver had the highest haemosiderin concentration. It contained more iron than the total amount found in the normal body.

Three livers, specially assayed, contained more iron than the total contribution of transfused iron. The source of this extra iron is uncertain, but may be the intestine. Five livers were diffusely fibrosed. The cause of this fibrosis was not the presence of the haemosiderin, and it is possible that a local nutritional factor was involved.

A. Wynn Williams.

Malignant Testicular Tumours, Particularly Chorionepithelioma, and the Possibility of Spontaneous Cure of Primary Testicular Teratoid, with a Note on Diabetes Insipidus. ROTH, F. (1950). *Z. Krebsforsch.*, **57**, 21. Bibliography.

From a study of 9 cases of testicular tumour the author concludes that seminoma is a "teratoid" tumour, and that all malignant tumours of the testis are related "teratoblastomata." Chorionepitheliomatous tissue is more often present in malignant teratomas than has been supposed. A case of rupture of the spleen due to metastases of a testicular chorionepithelioma in a man of 42, and another of disseminated chorionepithelioma with retrogression of the primary testicular tumour (like those reported by Prym, Michel, and others) and accompanied by gynaecomastia, are described, and the hormonal effects of testicular tumours are reviewed and discussed. In the latter case the patient had also had diabetes insipidus, which necropsy showed to be due to a coincidental chronic inflammatory lesion of the pituitary. *R. A. Willis.*

Genital Tract Tumours of an Adenomatoid Nature. WYATT, J. P., and KHOO, P. S. H. (1950). *Brit. J. Urol.*, **22**, 187.

An unusual type of epididymal tumour was described in 1941, and the case records of two additional specimens of similar tumours are now given. These tumours have been variously regarded as of endothelial, mesothelial, epithelial, and mesonephric origin. They are attached to, though not always part of, the epididymis. They are white or light brown, glistening, and whorled, like small fibromata. The histological appearances vary somewhat in different cases, and it is in the interpretation of these variations that the difficulties in diagnosis have arisen in reports of cases. The indefinite term "tumour of adenomatoid nature" should be retained until such time as the precise pathology is determined by the accumulation of evidence from succeeding cases. The tumours are not malignant. Simple local excision sufficed in all cases reported for complete recovery.

[Photographs of the tumours in the cases reported are reproduced.] *James Kemble.*

Nonchromaffin Paraganglioma of Ganglion Nodosum, Carotid Body, and Aortic-arch Bodies. LATTES, R. (1950). *Cancer*, **3**, 667.

An excellent account is given of 4 cases of tumours of the carotid-body type in unusual situations. In the first case the growth arose

from the ganglion nodosum of the vagus nerve; in the second there were two separate tumours, one arising in the vagus nerve and the other in the carotid body of the opposite side; the third patient had an upper anterior mediastinal tumour; and in the fourth case there were three independent tumours, involving respectively the ganglion nodosum, carotid body, and adventitia of the aortic arch. Microscopically, these tumours all resembled those of the carotid bodies and those of the glomus jugulare. The carotid body, the glomus jugulare, the paraganglion intravagale, and the aortic-arch bodies all have a similar structure and are probably similar chemoreceptors; they form a " paraganglionic system " which is quite distinct from the chromaffin tissues. The development of similar, and not infrequently multiple, tumours from these several structures is thus readily understandable. This is the first report of tumours of the aortic bodies in man, though Bloom has described such tumours in the dog (*Arch. Path.*, 1943, **36**, 1). The present paper contains a full bibliography, and also includes some excellent photomicrographs of the aortic and vagal paraganglia. *R. A. Willis.*

Prognosis in Carcinoma of the Breast. BLOOM, H. J. G. (1950). *Brit. J. Cancer*, **4**, 259.

A series of 470 cases of carcinoma of the breast treated at the Middlesex Hospital were graded according to the degree of tubule formation, uniformity in size, shape, and staining of the nuclei, and the number of hyperchromatic nuclei and mitoses.

By combining this method with the Manchester system of classification, a more accurate prognosis can be given than by classification alone. Certain tumours, if they are of a low histological grade, may be associated with a better prognosis even if they have reached Stage III than other more malignant grades still confined to the breast in Stage I; this finding is a strong argument in favour of the au hor's system of grading.

Possible fallacies in grading and staging are discussed.

Errors in staging arise from involvement of lymph nodes (as in the internal mammary chain) not revealed clinically or post-operatively; and from the fact that lymph nodes clinically enlarged may not be actually involved in tumour, and conversely that nodes involved may not be palpable clinically. Supraclavicular lymph-node involvement, undetectable either clinically or after operation, is another factor which might vitiate the accuracy of staging. Nevertheless, the author concludes that the system which he proposes is the best possible means of classification available, and he makes out a well-argued case that the universal adoption of this combined clinico-pathological method will lead to more accurate assessment of different methods of treatment.

H. J. B. Atkins.

Unilateral Renal Cortical Necrosis and Unilateral Benign and Malignant Nephrosclerosis associated with Contralateral Artery Occlusion: Report of a Case. ARONSON, S. M., and SAMPSON, M. C. (1951). *Arch. Path.*, **51**, 30.

The case is described of a man of 52 who complained of cough, fever, headache, and loss of weight. At necropsy the left kidney showed the changes of benign and malignant hypertension and gross cortical necrosis. The right kidney was normal. The renal artery on the right side was grossly sclerotic, with marked reduction of the lumen, which was terminally thrombosed. There were old and recent infarcts in the heart, a non-tuberculous cavity in the lung, and [?] viral hepatitis. The pituitary gland was normal.

It is thought that the hypertension was due to ischaemia of the right kidney, and that this was unaffected by the cortical necrosis because its functional activity was low and its nutritional demands adequately supplied by the capsular anastomotic vessels.

D. M. Pryce.

Correction.—Dr. Callender writes that in her paper " The Effect of Citrovorum Factor (Folinic Acid) on Megaloblasts *in vitro* " (*J. clin. Path.*, **4**, 204) the abbreviation $\mu\mu$g. was used for millimicrograms instead of mμg.

J. clin. Path. (1951), **4**, 393.

FINDINGS FROM LINGUAL SCRAPINGS TAKEN DURING TREATMENT WITH CHLORAMPHENICOL AND AUREOMYCIN

BY

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In a previous paper (Tomaszewski, 1951) a survey was made of the side-effects of chloramphenicol and aureomycin treatment. These were divided into general, gastro-intestinal, genito-rectal, and oral manifestations. Changes in the oral cavity were found more frequently than in other parts of the body. They consisted of dryness of the mouth, throat manifestations, angular stomatitis, and changes in the oral mucous membranes. Striking changes were seen in the dorsum of the tongue ; the most common was the disappearance of the normal whitish coating with some degree of atrophy in the filiform papillae, and in extreme cases marked atrophic glossitis with redness and soreness of the tongue. However, cases were also observed in which there was hypertrophy of the mucosa (hypertrophic glossitis) with overgrowth of the filiform papillae and brown discoloration. This discoloration was also sometimes seen in cases with some degree of atrophy of the papillae.

The present paper describes in detail the miscroscopical examinations of the lingual scrapings. The findings are correlated with the pathological changes which develop in the tongue during chloramphenicol and aureomycin therapy. The investigations were made on 126 patients treated with these antibiotics for various infective conditions in the wards of the Royal Infirmary, Edinburgh.

Method

Scrapings from the tongue were made at two- to three-day intervals, before, during, and for some time after treatment. The tongue was scraped with a sterile slide from as far back as possible, and the material ground with another slide in order to obtain an even film. After heat-fixation the film was stained with Gram or, in certain cases, with other stains. The scraping procedure is simple and makes it possible to follow the action of antibiotics on microflora and on the epithelium of the tongue. Microflora can thus be studied in their natural environment.

Results

Scrapings from the tongue usually contain epithelial cells, microflora, occasional leucocytes, and amorphous debris.

Changes in the Tongue.—In the majority of patients undergoing treatment a typical series of changes can be seen. The intensity of these depend very much on the

dose of the antibiotics and the duration of treatment. The previous use of penicillin or streptomycin must be considered. The usual dose used in the study was 2 g. daily. In certain cases small doses, 1 g., or higher doses, 3–4 g. daily, were used. Vitamin B complex was given to a group of patients simultaneously with the antibiotics to study the effects of these vitamins on the onset and development of the oral lesions.

In a few days, in some instances 24 to 48 hours after the start of antibiotic treatment, the bacterial flora disappear in the majority of cases and are replaced by fungous flora. At the beginning of treatment there is usually an increased shedding of epithelial cells. Leucocytes often appear in increasing amounts, especially in cases with marked atrophic glossitis. A typical series of changes, occurring during aureomycin treatment in a patient aged 20, who developed atrophic glossitis with soreness and redness of the tongue, is shown in Figs. 1–6. These illustrations refer to a patient who showed a normal tongue before treatment. In a few days shortening of the papillae was noted, and was followed by definite denudation, redness, and soreness of the tongue, and by angular stomatitis. At the end of the three weeks' treatment a slight, brown discoloration was visible on one side of the tongue. The surface of the tongue did not change with the sudden reappearance of the bacterial flora, which took place while the patient was still continuing treatment. After stopping treatment the angular stomatitis and denudation persisted for about two weeks.

Before treatment the tongue scrapings showed a predominance of Gram-positive cocci and also some Gram-positive bacilli (Fig. 1). Five days after the start of treatment (Fig. 2) a few yeast cells (*Candida albicans*) were seen showing the development of some mycelium elements ; bacteria apparently were completely absent. At a later stage (Fig. 3) the yeast and mycelium elements were more abundant ; bacteria were absent. At that stage large numbers of leucocytes were present (Fig. 4). Clinically, denudation, redness, and soreness of the tongue had developed. By the twelfth day, while the patient was still undergoing treatment, bacterial flora had developed on the tongue, and appeared uniformly diplococcal in character (Fig. 5). At this stage a diminished amount of yeast cells could still be seen. On the last day of treatment (Fig. 6) the tongue scrapings showed very rich, practically uniform coccal flora and a few chains of bacilli. The daily dose was 2 g., the total dose 44 g.

Epithelial Cells.—The number of epithelial cells alters during treatment. Epithelial cells are usually found alone, but sometimes they are seen in plaques of two, three, or more cells. The nucleus is usually well defined, but sometimes it is absent from cells showing degenerative changes.

In addition to the usual epithelial cells other cells are occasionally found. Some are small and navicular and stain deeply. Another type shows peculiar deep, red-violet blotches of various sizes, which accumulate and give to these cells an extraordinary appearance, resembling the spots of a leopard skin (Fig. 7). These " blotchy cells " appear normally only in a certain percentage of persons, but sometimes increase in number in the course of antibiotic treatment. Differential staining with the Shorr method (Shorr, 1941) shows that they are keratinized epithelial cells. However, the nature of these " blotchy " changes is unknown.

Shedding of the epithelial cells is most marked during the first days of treatment. As the tongue becomes denuded and the filiform papillae shrink, the epithelial cells

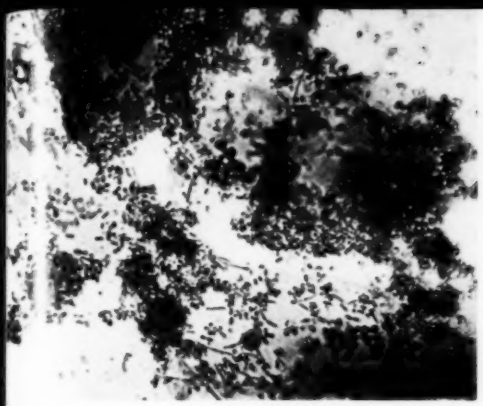

FIG. .—Scraping of the tongue before aureomycin t atment; profuse mixed bacterial flora (× 1,000). C inically normal appearance of the tongue.

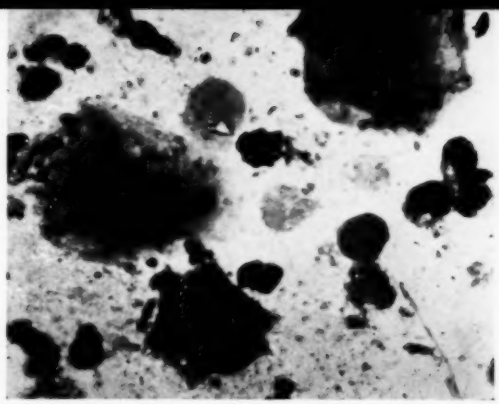

FIG. 4.—Same scraping as in Fig. 3; absence of bacteria; many leucocytes (× 500). Clinically redness and soreness of the tongue.

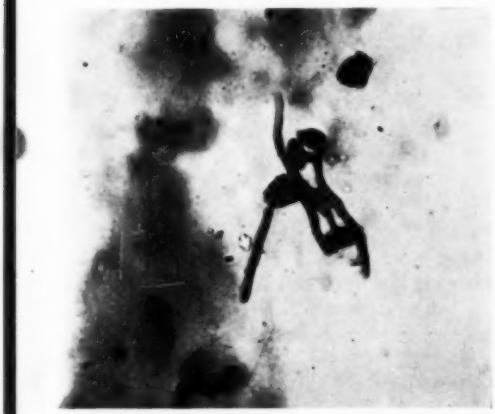

FIG. 2.—Fifth day of treatment; absence of bacteria; first yeast-like fungi to be seen (× 1,000). Clinically denudation of the tongue.

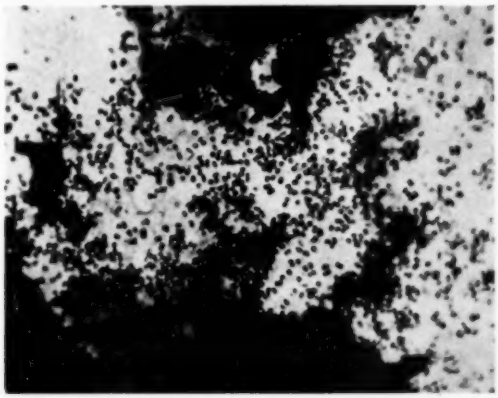

FIG. 5.—Twelfth day of uninterrupted treatment; sudden appearance in 24 hours of a rich, uniform coccal flora, probably an aureomycin-resistant strain; disappearance of fungi (× 1,000). Clinically still redness of the tongue.

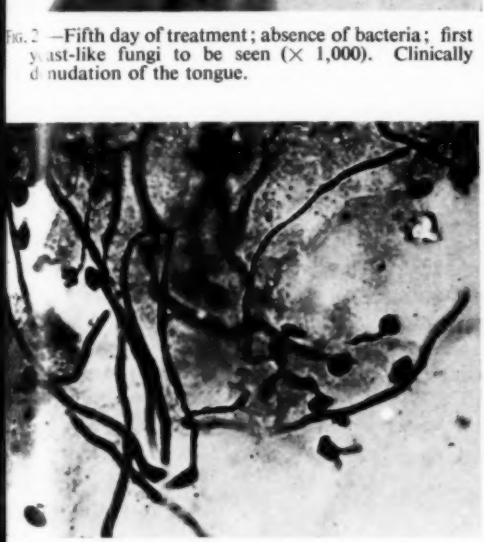

FIG. 3.—Seventh day of treatment; absence of bacteria; rich growth of fungi, mostly mycelium; few ovo yeast cells (× 1,000).

FIG. 6.—Twenty-first day of treatment; bacterial flora continuing, now more of a mixed type (× 1,000); absence of fungi. Clinically some improvement of the tongue surface. Treatment stopped on that day.

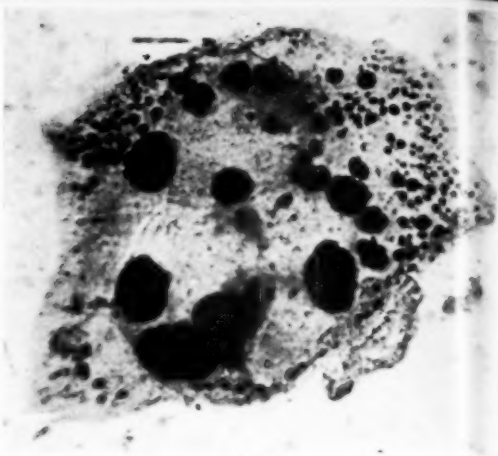

FIG. 7

FIG. 7.—"Blotchy" epithelial cell; nucleus clearly visible (× 1,000). Gram-stained. Treatment with chloramphenicol.

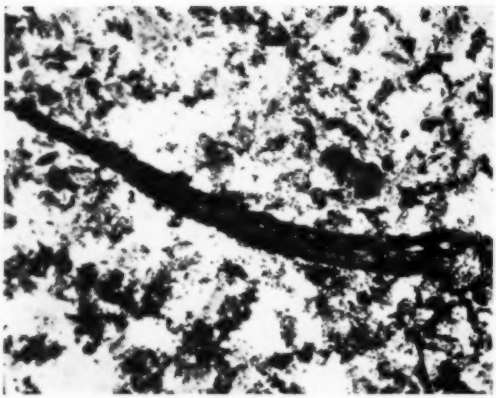

FIG. 8

FIG. 8.—Broken fragment of filiform papillae; many epithelial cells (× 65).

become scarcer, and it is increasingly difficult to obtain sufficient material for investigation. Occasionally broken fragments of filiform papillae may be seen as long bodies pointed at one end (Fig. 8). Their number usually increases with treatment in cases showing hypertrophic glossitis.

Bacterial Flora.—Bacterial flora of the tongue, observed during antibiotic treatment, nearly always show a change in character and in number. In the majority of cases (59%) the bacterial flora rapidly diminish in number, and usually disappear completely in a few days. In a number of patients, usually those who receive smaller doses of the antibiotics, bacteria are found throughout the course of treatment, but are markedly reduced in number and frequently change in character. In a few instances there is a change from mixed flora to a growth which is predominantly coccal.

In the majority of cases the absence of bacteria persists for several days after treatment has been stopped. The first bacteria to reappear in large numbers after the discontinuation of treatment are usually cocci. The bacterial flora are not always of the same type as before treatment, and are often more uniform in character.

An interesting observation was that scrapings might show complete absence of bacteria one day and new rich bacterial flora of a uniform character 24 hours later, while the patient was still undergoing treatment (Fig. 5). In three cases (two treated with aureomycin, one with chloramphenicol) a pure rich culture of cocci developed. This phenomenon is most probably due to the development of a chloramphenicol- or aureomycin-resistant strain.

Fungous Flora.—In the majority of cases yeast cells are first found 24 to 48 hours after the start of treatment. Their number increases with the progress of treatment and with the rapid disappearance of bacteria. The growth of fungi is sometimes very profuse, dominating the whole picture.

Fungi were found in 67% of tongues examined, mostly in cases with complete absence of bacteria, though in about one-fifth of the cases fungi coexisted with bacteria. There were cases with no visible fungi. However, if fungi are not seen in several consecutive scrapings it is not an absolute proof of their absence, since their presence may be confirmed on culture. All stages of fungus development may be seen: at first a few scattered yeast cells, and later mycelium and oval yeast cells, which often appear as large clumps (Fig. 9). After treatment is ended fungi usually persist for several days, and sometimes for as long as several weeks. In all cases in which the type of fungus was identified, it proved to be *Candida albicans* (*Monilia*). This agreed with the observations of other authors (Harris, 1950; Williams, 1950; Woods, Manning, and Patterson, 1951).

FIG. 9.—Clump of fungi; mycelium and yeast cells matted together; absence of bacteria (× 500). Treatment with chloramphenicol.

Leucocytes.—A few white cells, scattered among the epithelial cells, are often found in normal scrapings. They are usually dead cells, showing marked degenerative changes, the contour of the nucleus being hardly recognizable and only the outlines of the cells visible.

During treatment with chloramphenicol and aureomycin the number of leucocytes frequently increases, and their nucleus is usually well defined (Fig. 4). The increase of leucocytes is most marked in those cases which show atrophic glossitis with soreness and redness, but it is also encountered in patients whose tongues are either normal in appearance or have a brown coating.

Amorphous Debris.—Masses of amorphous debris are commonly seen in the films. They are composed of particles of food and of decomposed cellular material. There is sometimes a large quantity of mucus, and an excess is found in patients who complained of marked dryness and a "sticky" feeling in the mouth.

The Relation of the Microscopical Findings to the Lingual Changes

A number of persons showed an apparently normal tongue during the antibiotic treatment. In such patients the bacterial flora were present, but diminished in number and changed in character. In a number of cases the coexistence of bacteria and fungi was noted. A high percentage of patients in this group had received vitamin B complex simultaneously with the antibiotics. The tablets used in this study contained aneurin, nicotinic acid, and riboflavin. It must be emphasized,

however, that in a few cases acute oral lesions, resembling avitaminosis B, especially ariboflavinosis, developed quickly in spite of vitamin treatment.

The denudation of the tongue, which took place in the first few days of treatment, was associated with a rapidly diminishing number of bacteria. Once the whitish coating has disappeared, atrophic changes may follow.

In atrophic glossitis there were usually a few fungi, although in a few cases there was a substantial growth. Bacteria were mostly absent or very sparse. An increase in leucocytes was common.

In hypertrophic glossitis there were nearly always many fungi, no doubt because of the better conditions of growth. Bacteria as a rule persisted in diminishing numbers during treatment, but occasionally disappeared, to reappear in a few days.

Brown discoloration of the tongue (melanoglossia) was usually associated with hypertrophic glossitis. In a few cases, however, it could be seen in a partly atrophied dorsum. The scrapings from brown ("black") tongues usually revealed large numbers of fragments of the papillae, which appeared brown in unstained films. No particular type of bacterial flora was found, nor any fungi, other than of the *Candida* type. It must be emphasized that there were a few cases of melanoglossia where no fungi were detected.

The results of examination of the lingual scrapings and their relationship to the macroscopical appearance of the tongue are shown in Table I.

As there was no significant difference in the development of the oral changes in cases treated with chloramphenicol and aureomycin, the results are given jointly.

TABLE I

MICROSCOPICAL CHANGES IN LINGUAL SCRAPINGS IN 126 CASES TREATED WITH CHLORAMPHENICOL AND AUREOMYCIN

Appearance of the Tongue	Total No. of Cases	Absence of Bacteria (No. of Cases)	Percentage* of Total Cases	Presence of Fungi (No. of Cases)	Percentage of Total Cases	Increase in Leucocytes (No. of Cases)	Percentage of Total Cases
Normal	30	5	16	13	43	9	30
Denuded or atrophic	83	68	81	60	72	38	45
Brown, and hypertrophic glossitis ..	13	2	15	12	92	4	30
Total ..	126	75	59	85	67	51	40

* There was a considerable overlap in the incidence of absence of bacteria, presence of fungi, and increase of leucocytes. Hence the sum of the percentages in each column is more than 100.

Discussion

The devastating effect of the antibiotics on the bacteria can be seen not only in the oral cavity, but also in the upper respiratory tract (Gray, 1950; Meads, Rowe, and Haslam, 1951), in the intestines, and in the external genitalia. An upset of balance between bacteria and host occurs, and as a result certain functional and anatomical changes develop in the tongue and are reflected in the lingual scrapings.

It is possible that the bacteria exert some influence on the shedding of the squamous epithelial cells which cover the filiform papillae. In the absence of

bacteria the shedding becomes scanty, the normal whitish coating disappears, and denudation and redness of the tongue follow. The filiform papillae shrink and sometimes actually atrophy.

Antibiotics may exert a chemical action on cell metabolism. Recently Lépine, Barski, and Maurin (1950) investigated the action of chloramphenicol and aureomycin on the proliferation of normal cells (fibroblasts, epithelial cells, tissue from mesencephalon) in tissue cultures. They found that chloramphenicol and aureomycin, in doses which are within the range of blood concentration in treated persons (10 γ/ml.), could retard the rate of growth and exert an injurious effect on proliferating normal cells. Cells from cultures containing 100 γ/ml. showed vacuoles and fatty degeneration. Fibroblasts were more readily inhibited than epithelial cells. Although it has not yet been proved that these observations are applicable to mature cells *in vivo*, nevertheless such a possibility must be taken into account in attempting to explain atrophic changes in the lingual mucosa. It is of interest to note that aureomycin produces fatty changes in liver (Yesner and Kunkel, 1951; Lepper, Wolfe, Zimmerman, Caldwell, Spies, and Dowling, 1951). Whether the appearance in increasing numbers of "blotchy" cells is connected with such a chemical action of antibiotics is an open question. It may be significant that when these antibiotics (and penicillin) are given as lozenges the number of these cells is sometimes greatly increased in persons normally showing small numbers of such cells (unpublished data).

It has been mentioned before that some patients were treated simultaneously with vitamin B complex and antibiotics. In these patients the tongue retained its normal appearance during the treatment more often than those who did not receive vitamin B complex. However, in a few cases a red, sore tongue and angular stomatitis developed rapidly in spite of vitamin treatment. Similar results were observed by Leitner (1950), Hobson and Rice-Oxley (1951), and others. The striking similarity of the oral changes associated with antibiotic therapy to those of vitamin B deficiency (especially ariboflavinosis) has inclined some authors to the view that these lesions are in fact signs of vitamin B deficiency (Harris, 1950, and others) produced by the destruction of the intestinal flora by antibiotics. As the mucous membrane manifestations appear sometimes within 48 to 72 hours after the start of combined antibiotic and vitamin treatment, it is somewhat difficult to explain this sudden and acute vitamin B deficiency. There is some doubt as to the interpretation of these oral changes as signs of true vitamin B deficiency. It is known (Gilder, 1950) that it takes a considerable time to produce definite deficiency symptoms in well-nourished and healthy subjects. It is also puzzling, as Leitner (1951) states, that 10–20 times the daily requirements of vitamin B complex are necessary to prevent or alleviate the deficiency symptoms after a short course of antibiotic treatment in well-nourished persons.

It is possible that a more complicated mechanism might perhaps play a role in the production of the oral (and recto-genital) changes; for instance, some chemical action of the antibiotics on the tissue (? action on enzymes), monilial infection, or anti-vitamin (Kodicek, 1949). Recently a suggestion has been put forward (Gewin and Friou, 1951) that aureomycin and chloramphenicol might exert a competitive blocking action on intracellular metabolism and thus produce changes resembling

vitamin deficiency. Studies of the metabolism of the various vitamins of the B group during antibiotic treatment might shed some light on this problem.

There are so far some indications that penicillin at least may produce in man certain alterations in the content of vitamins C, B, and riboflavin, resulting in some deficiency state (Mosonyi and Oblatt, 1948).

The invasion of the oral cavity by *Candida* (*Monilia*) *albicans* during treatment is an undesirable side-effect, which has been reported by a number of workers (Zimmerman, 1950 ; Harris, 1950 ; Williams, 1950 ; Woods *et al.*, 1951 ; Moore, 1951). *Monilia*, although usually harmless, may in certain conditions acquire pathogenicity. In some of our patients monilia were found not only in the lingual dorsum but also in the crusts of angular stomatitis, in rectal and vaginal discharges, and in large amounts in the stools.

In healthy persons, even before treatment, single yeast cells are occasionally present in scrapings of the tongue. A competitor of bacteria is therefore constantly present in the oral cavity, and takes the place of the bacterial flora when the latter disappears. It seems that some kind of antagonism exists between oral bacteria and oral fungi. The presence of rich bacterial flora prevents the growth of fungi. In some cases, however, bacteria and fungi have been found to coexist, for a certain time at least. Although Zimmerman suggests some stimulating action of the antibiotics on the growth of monilia, Woods and his colleagues deny it on the strength of experiments *in vitro*.

The increase of leucocytes in the scrapings during antibiotic treatment suggests some inflammatory process in the tongue. It was nearly always associated with the presence of fungi and with the absence of bacteria. In very few instances where the number of leucocytes was increased were fungi found coexisting with bacteria, and only in one case were bacteria present in the absence of fungi. The association of an increased number of leucocytes with fungi may be regarded as evidence of an inflammatory reaction to the fungous invasion.

The results indicate that the existence of normal bacterial flora is necessary for the healthy structure and function of the mucous membranes. Experiments on germ-free animals do not prove that the absence of normal bacterial flora actually provokes any pathological changes in the mucous membranes (Reyniers, Trexler, and Ervin, 1946, and Reyniers, Trexler, Ervin, Wagner, Luckey, and Gordon, 1949a and b). It may therefore be that the bacteria exert a protective action, defending the mucous membranes against invasion by fungi.

Summary and Conclusions

Examination of scrapings from the tongue has been carried out in 126 patients receiving treatment with chloramphenicol and aureomycin.

In the majority of patients these antibiotics cause the disappearance of the normal bacterial flora. In the remainder there is a marked diminution in the number, and sometimes also in the character, of the bacteria. Occasionally the appearance of the new strain of bacteria, suspected to be chloramphenicol- and aureomycin-resistant, was noted in the course of treatment.

Epithelial cells are abundant in the first days of treatment. Peculiar, " blotchy " epithelial cells are sometimes seen in increased numbers during the treatment.

In a great number of patients fungous flora (yeast-like fungi, usually *Candida albicans*) replace the bacterial flora. The fungi are profuse in hypertrophic glossitis, and scanty in the atrophic type. In a few cases absence of bacteria as well as fungi has been noted. An antagonism between bacterial flora and fungi in the oral cavity is suspected. The increased number of leucocytes, which is particularly marked in atrophic glossitis, is most probably a sign of inflammatory reaction to the fungal infection.

The similarity of the oral lesions produced by the antibiotics to those of vitamin B deficiency is discussed, and the role of the bacterial flora in protecting mucous membranes is emphasized.

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THE ASSOCIATION OF EOSINOPHILIC POLY-ARTERITIS, LIBMAN-SACKS ENDOCARDITIS, AND ASTHMA WITH DIFFUSE COLLAGEN DISEASE

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In the last decade a considerable number of papers have been written describing fatal illnesses in which the salient clinical features were asthma, eosinophilia, and heart failure. Weir (1939), Harkavy (1941), Chafee, Ross, and Gunn (1942), Miller and Daley (1946), Bergstrand (1946), Smith (1948), and Lennox (1948) have all reported such cases, the clinical course of which differed little from the comparatively common asthma ending in heart failure or occasionally status asthmaticus. The post-mortem findings, however, showed lesions which linked those cases with the so-called collagen diseases or allergic syndromes.

In view of the great interest in collagen diseases at present the following case seems to be worthy of record.

Case History

A married woman, aged 36, was admitted to hospital on October 4, 1950, complaining of a rash of four days' duration.

She had been well until the beginning of her pregnancy in November, 1948, when she developed spasmodic attacks of asthma with bronchitis after she had had some teeth extracted.

She had a normal delivery in another hospital on July 4, 1949, and five days later developed a severe attack of asthma. She remained in hospital for ten weeks, and during this time the leucocyte count was 17,000, of which 2,500 were eosinophils. A radiograph of the chest was normal. Since then she had had recurrent asthmatic attacks lasting from five minutes to two days, with greenish, frothy sputum, occasionally blood-stained. She had frequent night sweats and lost much weight. There was increasing dyspnoea on exertion, and for two weeks before admission she had pains in the back, neck, and arms, with tingling in the hands. On September 30, 1950, she developed a whitlow of the right index finger and bronchitis. Her doctor gave her an injection of procaine penicillin, 600,000 units, and that evening she developed a diffuse purpuric rash.

On admission on October 4 her colour was good and she was not dyspnoeic. A petechial rash covered the trunk and was present in the mucous membranes of eyes and mouth. A few scattered rhonchi were heard in the chest, but there was no expiratory wheeze and no signs of emphysema. The pulse was regular, the heart not clinically enlarged, and there were no murmurs. The systolic blood pressure was 110, the diastolic indefinite. The liver and spleen were not palpable, but there was slight tenderness in the

upper abdomen. There was also some tenderness of the spinal muscles in the mid-dorsal region. The central nervous system showed no obvious abnormality.

During the next few days she had no asthmatic attacks, but there was slight pyrexia, the maximum being 100.4° F., and the pulse varied between 70 and 100.

Investigation of the blood showed mild normocytic anaemia, with a haemoglobin of 11.5 g. % and leucocytosis of 35,000 per c.mm., of which 15,500 were eosinophils. Blood platelets and bleeding and clotting times were normal. The erythrocyte sedimentation rate on October 11 was 38 mm. (Wintrobe, corrected).

The sputum contained many eosinophils but no Charcot Leyden crystals; *Strep. viridans* was grown on culture.

The urine contained a trace of albumin, a few leucocytes and hyaline casts, but was sterile on culture. The stools were positive for occult blood.

A blood culture (penicillinase added) taken on October 9 grew after five days a haemolytic streptococcus belonging to Lancefield's Group D.

X-ray examination of the chest on October 7 showed clear lung fields with a borderline cardiac enlargement. The pear-shaped outline suggested a pericardial effusion.

In view of the purpuric rash an inquiry was made about the various drugs with which the patient had been treated since the onset of asthma. These included ephedrine, "franol," "felsol," and "dormiprin." Iodides had produced a rash and had been discontinued. She had several courses of penicillin injections, the last in January, 1950, eight months before the onset of the present illness. There was no history of any reaction to these injections. A further course of penicillin was given from October 4 to 9.

On October 10 the purpuric rash had begun to fade, but the patient's general condition deteriorated. She complained of abdominal pain and vomited. The liver was palpable. Next day the pain was at the lower end of the sternum, and a pericardial friction rub was heard. There was congestion of the lung bases, but no engorgement of the neck veins or oedema of the ankles. Splinter haemorrhages were found under the nails in both hands. She died suddenly and unexpectedly on the morning of October 12.

Post-mortem Report

The body was that of a small, thin woman, with a fading purpuric rash on the trunk and limbs, splinter haemorrhages under the finger-nails, and a recent infection of the nail-bed of the right index finger.

Chest.—The pharynx, larynx, trachea, and oesophagus were normal. The pericardium was distended with about 500 ml. of clear, deep yellow fluid. The heart (321 g.) was small, and the entire surface covered with small flakes of fibrinous exudate. Apart from some dilatation of the left ventricle with thinning of the wall, the chambers were not enlarged. The endocardium of both auricles showed fairly diffuse thickening with yellowish verrucose vegetations, varying in size from 1 to 5 mm., scattered over the surface. The endocardium of the ventricles showed only slight patchy thickening, but numerous similar vegetations were found on the upper parts of the walls under the cusps of mitral and tricuspid valves, the distribution described by Libman and Sacks (1924). The largest vegetations had formed at the junction of the valve and ventricular wall (Fig. 1). The free margins of the cusps showed no obvious lesion. The pulmonary and aortic valves were normal. Small, pale, sub-endocardial flecks were seen in the interventricular muscle of the left ventricle, and the cut surface of the myocardium showed deep red and yellow mottling. The coronary arteries were patent. The aorta was healthy. Both lungs were slightly adherent at the apices posteriorly, and a small quantity of free fluid was present at the base of each pleural cavity. The lung substance was well aerated. A few small haemorrhagic areas were found in both lower lobes. There were no large infarcts. The bronchi were clear.

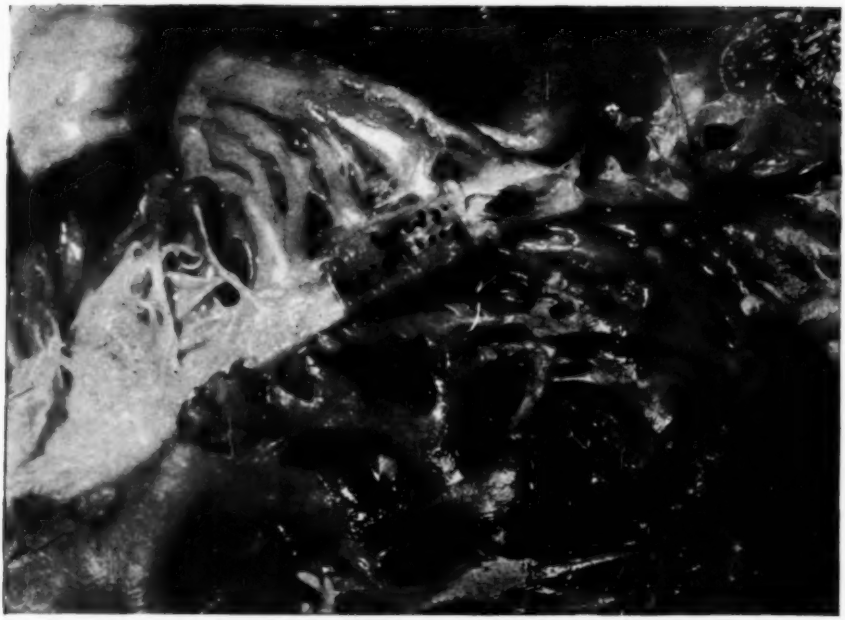

FIG. 1.—Right ventricle showing endocardial vegetations, × 2. The reflected tricuspid is seen.

Abdomen.—The liver (1,654 g.) was congested, with subcapsular haemorrhages producing a mottled surface. The spleen (153 g.) was firm and congested, with a few small infarcts. A splenunculus (1.5 cm. diameter) was present. The kidneys (left, 150 ; right, 146 g.) were congested. The capsules stripped easily. There were no definite infarcts. The uterus appeared normal. There was a small cyst of the right ovary. The pancreas was firm and congested. The other organs showed no obvious abnormality.

Head.—The brain (1,206 g.) was congested and oedematous. There were no obvious lesions.

Histology

Heart.—There was diffuse eosinophil infiltration of the myocardium, not only in the fibrous septa but between the muscle fibres. Many of the small arteries were thrombosed (Fig. 2) and surrounded by eosinophils. Focal granulomata with giant cells were also present in the perivascular connective tissue ; in some cases the granulomata were closely associated with, and apparently part of, the arterial wall, with giant cells forming from the intima (compare Fig. 8). Similar small granulomata were found in the muscle of the auricles not obviously connected with vessels, and giant cells appeared to be forming from degenerate muscle cells. In some areas there was marked fibrosis of the myocardium (Fig. 3), with focal degeneration of muscle fibres. There was also considerable infiltration of the endocardium and pericardium with eosinophils and plasma cells. Subendocardial areas of necrosis were present, surrounded by well-formed fibrous tissue resembling healing rheumatic nodules. Similar nodules and granulomata were found in the pericardium. Abacterial thrombi, consisting of fibrin and degenerate eosinophils

FIG. 2. — Myocardium showing diffuse eosinophil infiltration with thrombus in an arteriole. H. and E., × 90.

FIG. 3.—Fibrosis of myocardium with eosinophil infiltration. H. and E., × 400.

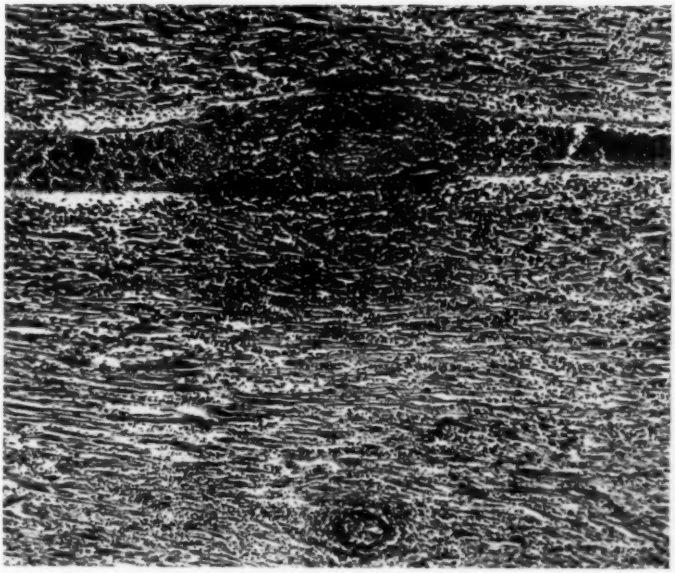

FIG. 2

FIG. 3

and polymorphs, were adherent to the thickened endocardium. Pyknotic nuclei were numerous in the degenerate muscle fibres and in the pericardial fat, but the haematoxylin-stained bodies described by Gross (1940) and Klemperer, Pollack, and Baehr (1941) in lupus erythematosus could not be found.

Lungs.—Diffuse eosinophil infiltration of alveoli and alveolar walls in the right and left lower lobes had produced focal areas of consolidation. Many of the small arteries were thrombosed, the thrombus containing numerous degenerate eosinophils (Fig. 4). The walls of the vessels showed no obvious change, and the elastic laminae were well preserved. The fibrinoid necrosis present in the splenic arterioles (Fig. 5) could not be found in any of the sections, and the walls of the patent arterioles appeared healthy. Many of the small bronchioles were contracted and plugged with mucus and eosinophils, and there was some hypertrophy of the peribronchial muscle.

Spleen.—The splenic pulp showed diffuse eosinophil infiltration with more marked cellular foci around degenerate or partially degenerate arterial walls. In Fig. 5 a patch of fibrinoid necrosis is shown in the longitudinal section of a small artery. There is a definite break in the wall with protrusion of the fibrinoid material across the lumen of the vessel. A ring of eosinophils surrounds this area. Similar lesions were found throughout the sections and, in some, heavy deposits of fibrin, staining deep blue with Mallory's phosphotungstic acid-haematoxylin, were present. Breaks in the elastic lamina were seen in sections stained by Weigert's method, resembling the lesion of polyarteritis nodosa (Pagel, 1951). Numerous small granulomata, consisting mainly of giant cells, were scattered throughout the pulp (Figs. 6 and 7). Some were paravascular in distribution, others appeared to be forming from vessel walls (Fig. 8). The vascular lesions were quite apart from the macroscopically infarcted areas. There was no increase in the fibrous tissue surrounding the penicillary arteries.

Liver.—There was marked chronic venous congestion, with fibrosis and eosinophil infiltration of the portal tracts. Some of the arterioles showed fibrinoid degeneration of their walls with the formation of a few small granulomata.

Pancreas.—In the connective tissue around the ducts there were small aggregations of giant cells, and some of the small arteries showed fibrinoid changes in their walls with eosinophil infiltration.

Kidneys.—There was diffuse congestion and small, wedge-shaped subcapsular areas of eosinophil infiltration throughout the cortex. These surrounded small thrombosed arteries. Many of the thrombi had become hyalinized and partly retracted from the wall. There was no obvious fibrinoid necrosis of the walls of the thrombosed vessels. The glomeruli were congested, but their capillaries appeared healthy, and no " wire loop " changes could be detected. Apart from slight interstitial fibrosis around the thrombosed arterioles and one definitely infarcted area, the remainder of the kidney tissue appeared normal, the tubular epithelium showing only post-mortem change.

Lymph Nodes.—There was congestion and oedema with diffuse eosinophil and plasma cell infiltration.

Skin.—Small foci of sub-epithelial haemorrhage were present with a few eosinophils, but no obvious lesions of the arterial walls.

Sternal Marrow.—Apart from a great excess of eosinophils and fairly numerous megakaryocytes there was nothing significant in the marrow.

Voluntary Muscle.—The section showed no apparent lesion.

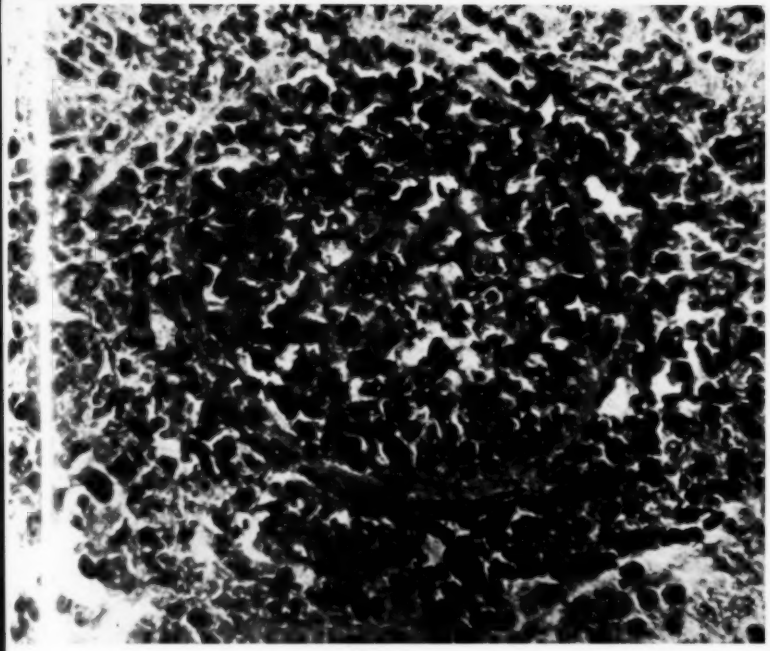

FIG. 4.—Section of lung showing eosinophil thrombus in arteriole. H. and E., × 400.

FIG. 5.—Section of spleen showing fibrinoid necrosis of arteriole with surrounding eosinophil infiltration. H. and E., × 400.

FIG. 4

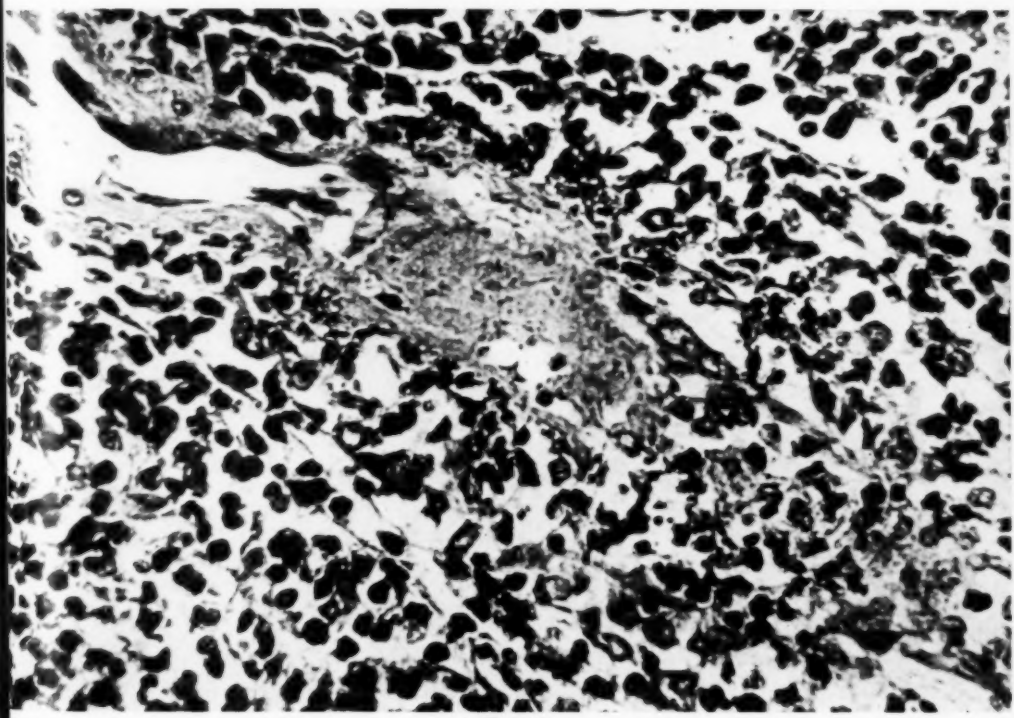

FIG. 5

FIG. 6.—Section of spleen showing small granulomatous areas. H. and E., × 90.

FIG. 7.—Section of spleen showing giant cell formation in granuloma. H. and E., × 400.

FIG. 6

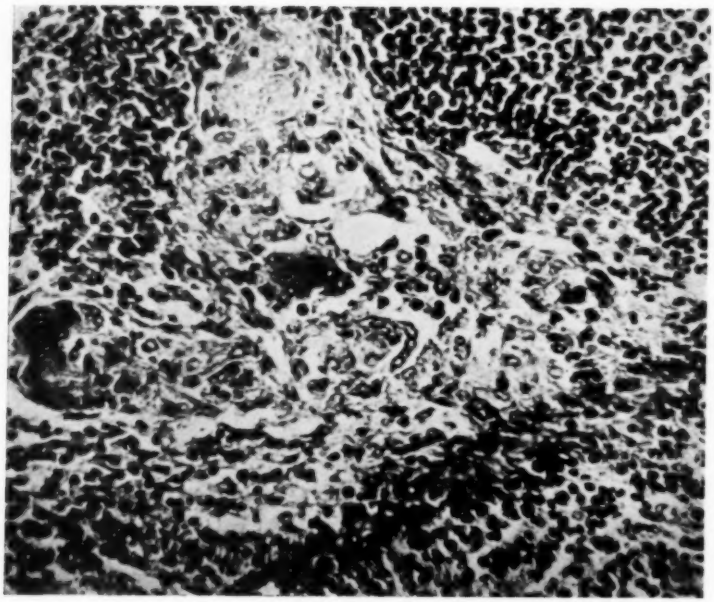

FIG. 7

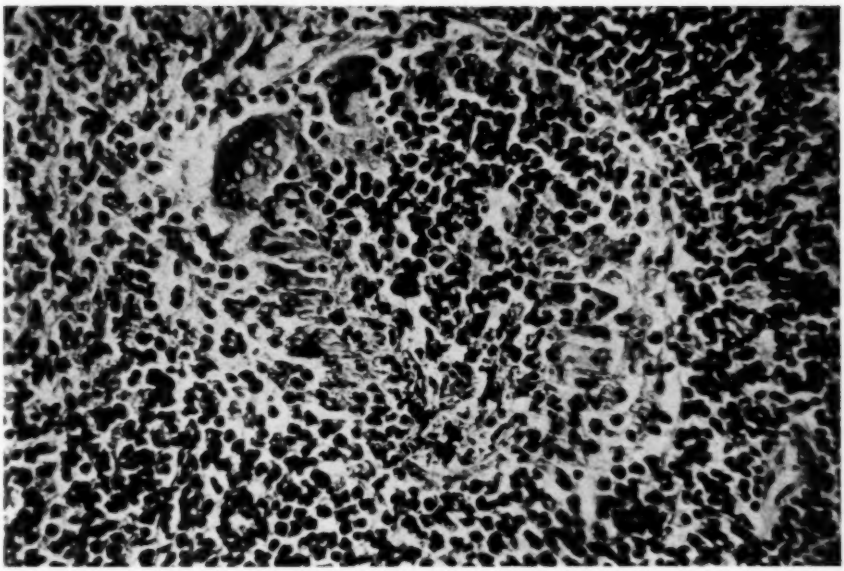

FIG. 8.—Section of spleen showing giant cells forming from intima of thrombosed arteriole. H. and E., × 400.

Discussion

The clinical course of this patient was very much the same as that of those previously described. The disease had apparently begun with asthma and bronchitis nearly two years before death. In Bergstrand's four patients asthma had been present from two months to two years. Lennox's patient had a history of asthma for five years and Smith's for about six months.

The purpuric rash and splinter haemorrhages shown by our patient, combined with pyrexia, strongly supported a diagnosis of bacterial endocarditis. The blood culture taken the day before death grew a haemolytic streptococcus Lancefield D after five days' incubation. Since by this time post-mortem examination had shown an endocarditis with vegetations of the typical Libman-Sacks distribution, and histologically the vegetations were abacterial, the streptococcus may be regarded as a contaminant. Haemolytic streptococci of Lancefield Group D are an unusual cause of subacute bacterial endocarditis, and, since the particular strain isolated was sensitive to penicillin, it is unlikely that the finding was of pathological significance. Cultures taken at necropsy grew only a non-haemolytic enterococcus.

The constant leucocytosis which was present, not only during the last part of the illness but also after the patient's confinement a year before, is also suggestive of a bacterial infection. The high proportion of eosinophils, however, which finally reached 16,000 per c.mm., favours the diagnosis of an allergic response to some antigen. Such high eosinophilic counts are unusual in uncomplicated asthma,

but of the papers already quoted Harkavy (1941) and Miller and Daley (1946) reported totals of 25,000 to 30,000 per c.mm. Similar high counts have been found by Lumb (1950) in eosinophilic arteritis, and referred to by Lennox (1948) in a series of cases of eosinophilic endocarditis. Although asthma was not a clinical feature in either of these diseases the histological changes closely resembled those found in the present case.

Histologically the main lesion is a diffuse arteritis with fibrinoid necrosis of the wall affecting the small vessels of the myocardium, spleen, and pancreas, and surrounded by eosinophils (Fig. 5). This is essentially the lesion found by Rich (1942) in serum sickness and sulphonamide sensitivity, and shown by the experiments of Rich and Gregory (1943) to be a manifestation of hypersensitivity in animals. The interstitial myocarditis with infiltration of polymorphs, monocytes, and eosinophils described by Rich (1942) in three of his patients with polyarteritis nodosa is an outstanding feature of the present case (Fig. 2). The pericarditis with plasma cell and eosinophil infiltration, and the endocardial changes with the formation of vegetations, are part of the same process. The extensive myocardial fibrosis appears to be of much longer duration (Fig. 3). As asthma and bronchitis were the first symptoms of the illness it might have been expected that the lung lesions would be more chronic in type, but in all the sections examined the changes appear to be comparatively recent (Fig. 4). This corresponds with the negative *x*-ray findings throughout the illness and the focal distribution of the lesions in both lower lobes. The changes in the lungs and kidneys suggest an embolic origin from the endocardial vegetations rather than a primary arteriolitis.

The other type of lesion consists of small granulomata found mainly in the spleen and adjacent splenunculus (Figs. 6 and 7). They have been described as "paravascular" by Pagel (1951), but in Fig. 8 giant cells are forming from the intima of a small splenic vessel. Bergstrand (1946) found intimal formation of giant cells, and an illustration in his paper shows similar changes to those in Fig. 8. These focal granulomata are also associated with hypersensitive states and are usually surrounded by eosinophils.

Although there was clinical and histological evidence of hypersensitivity it is difficult to decide the nature of the antigen or antigens responsible. The patient was known to be sensitive to iodine, and polyarteritis nodosa has been described by Rich (1945) following the administration of iodine to a sensitive subject. No iodine, however, had been prescribed since the patient's original allergic reaction. A bacterial antigen cannot be eliminated, since the onset of the illness followed tooth extraction, and exacerbations occurred during the puerperium and following a whitlow. From the history it seemed possible that penicillin was the offending substance, since the final dose apparently precipitated the final allergic response. Unfortunately no satisfactory skin tests were done before the patient died. Gold (1951) reported a case of lupus erythematosus which developed after penicillin injections, and several others which deteriorated following penicillin therapy, but no detailed pathological data were presented.

Further difficulty is encountered when an attempt is made to place this case in any definite disease category. Clinically the illness began with asthma and bronchitis two years before death. Apart from the severe attacks after the birth of her second child, there appears to have been nothing to distinguish the course of the disease from

any other case of asthma until the last few weeks of her life, when the symptoms suggest a widespread disease process affecting the cardiovascular as well as the respiratory system. The pathological findings confirm the widespread nature of the lesions, but do not correspond to any of the established syndromes. There is a diffuse arteritis, but the skin and muscle lesions found in polyarteritis nodosa were not present. The lung changes suggest a severe form of Löffler's (1936) benign eosinophilic alveolitis, and the endocardial changes could be linked with Löffler's endocarditis with eosinophilia. The Libman-Sacks distribution of the endocardial vegetations is usually associated with lupus erythematosus, but other findings in lupus erythematosis, such as the typical rash and the histological changes occurring in the glomerular capillaries and splenic arterioles, were not present. The conception of diffuse disease of fibrous tissue originated by Klinge (1930) in relation to rheumatic fever has been elaborated by many later writers, including Klemperer, Pollack, and Baehr (1941), and many diseases previously considered as entities have been correlated. Whatever the aetiology, in some cases allergic and in others without any obvious allergic basis, the fundamental lesion of fibrinoid necrosis of fibrous connective tissue with surrounding cellular reaction and the formation of granulomata is similar in all diseases of the group. Since the changes found in any one case must therefore depend upon the site of the lesions and the extent to which the organ is affected by the disease process, it is not surprising that clear-cut clinical and pathological differentiation does not always occur. The case described in this paper is an example of the overlapping of the syndromes grouped under the heading of diffuse collagen disease.

Summary

A fatal case, presenting as asthma with high eosinophilia, is described.

The pathological findings include arteriolitis, endocarditis, myocarditis, granulomata, with giant cell formation in the spleen, multiple changes in the lungs, and eosinophil infiltration of all the affected organs.

The post-mortem histological changes are discussed and linked with the allergic group of " collagen " diseases.

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SYMPTOMATIC HAEMOLYTIC ANAEMIA ASSOCIATED WITH OVARIAN TERATOMA IN A CHILD

BY

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Symptomatic haemolytic anaemia has been defined by Singer and Dameshek (1941) as a haemolytic syndrome often indistinguishable by haematological methods from the well-known picture of familial haemolytic jaundice, but showing a definite aetiological relationship to an underlying disease such as neoplasm, leukaemia, or Hodgkin's disease. The diagnosis of symptomatic haemolytic anaemia can only be accepted, as Davis (1944) points out, when there are reasonable grounds for concluding that the anaemic state results mainly, or exclusively, from accelerated red cell destruction, which in turn is conditioned by the development of an underlying morbid process. "In other words," Davis writes, "there must be evidence that the anaemia is of haemolytic origin, and that its onset was preceded by the underlying primary condition." Judged by these criteria, the diagnosis of symptomatic haemolytic anaemia in the case we are about to report cannot be disputed. There was every evidence of accelerated red cell destruction, and the ovarian teratoma must have originated before the anaemia, although its presence was not detected until the child became anaemic. A total remission of the haemolytic syndrome immediately followed the removal of the teratoma, a fact which strongly suggests an aetiological role on the part of the tumour in the causation of the anaemia. Such well-defined examples of symptomatic haemolytic anaemia are rare, and only four instances where the anaemia was associated with an ovarian tumour have hitherto been reported in the literature.

Clinical Features

A girl, aged 4 years 9 months, was referred to the General Infirmary at Leeds in April, 1950, with anaemia. Three months previously, at a routine visit to a welfare centre, pallor was noticed, for which iron was prescribed. She remained well until eight weeks before attending hospital, when she complained of pains in her limbs and was observed to be jaundiced. Her mother noted that the urine was dark and thick. No change had been noticed in the stools, which were black. After a week she began to improve, and by the end of six weeks she seemed to have recovered. Shortly afterwards, ten days before admission, the jaundice recurred, and this was associated with listlessness and anorexia, but there was no change in the colour of faeces or urine.

On examination the patient was seen to be a moderately well nourished, fair-haired girl weighing 34 lb. She was clearly anaemic and slightly jaundiced. The liver and spleen were not enlarged. A firm rounded mass, the size of a tangerine, was felt just above the pubis, and slightly to the right of the mid-line. It was not tender, and it could be moved slightly from side to side. The tumour was not palpable on rectal examination, and was not altered by repeated enemata and vesical catheterization. The external genitalia were normal. No other relevant findings were noted.

Laboratory investigations, details of which are given below, showed a severe haemolytic anaemia. The pelvis was examined radiologically, and a molar tooth with some adjacent irregular calcified shadows was seen in the position of the tumour. In view of the known association of ovarian teratoma with symptomatic haemolytic anaemia, and of the failure of splenectomy alone in some reported cases, it was decided to observe what effect the initial removal of the ovarian tumour had on the anaemia.

On May 20 a transfusion of 300 ml. of cross-matched Group O whole blood was given slowly. There was no untoward reaction. The blood haemoglobin was thereby raised, but signs of active haemolysis persisted. On May 23 the child received a further transfusion of 1,000 ml. of blood.

On May 24 Mr. A. J. C. Latchmore operated, and through a right paramedian incision removed a unilocular cystic teratoma (dermoid cyst) of the right ovary 7.5 cm. in diameter. After excision a tiny portion of the ovary remained proximal to the ligature. The left ovary felt normal.

Recovery from the operation was uneventful. On the third day the haemoglobin had fallen to 86%, but had returned to 100% on the fifth day (Table I). The patient was discharged on June 12, and when last seen four months later was symptom-free and in good health.

TABLE I

ANALYSES OF BLOOD COUNTS

Date	Hb (100% = 14·5 g./100 ml.)	R.B.C. (m./c.mm.)	Microspherocytes	Nucleated Red Cells per 100 W.B.C.	Reticulocytes (% of Red Cells)
Apr. 20 ..	44%	1·98	++	Occasional	39%
,, 21 ..	47%	—	++	5	—
,, 24 ..	48%	—	++	3	—
May 2 ..	49%	2·3	++	1	39%
,, 5 ..	58%	2·6	+	2	—
,, 13 ..	45%	2·05	+++	5	47%
,, 20 ..	Transfusion 300 ml. whole blood				
,, 24 ..	61%	2·7	++	1	33%
Operation ..	Removal of ovarian teratoma ..			Transfusion (1,000 ml.)	
,, 25 ..	100%	4·6	+	0	10%
,, 26 ..	86%	3·8	0	0	7·6%
,, 29 ..	100%	4·7	0	0	3%
June 3 ..	95%	4·5	0	0	2%
,, 21 ..	90%	4·3	0	0	0·3%
Oct. 10 ..	90%	4·7	0	0	0·8%

Laboratory Investigations

Haemoglobin and Red Cells.—Before treatment (Table I) Hb varied from 44% (6.38 g./100 ml.) on admission to 58% (8.41 g./100 ml.), and the red cell count from

1.98 to 2.6 m./c.mm. Both the red cell count and haemoglobin improved slowly after admission, but 10 days before operation there was a relapse.

Red Cell Size.—On May 5 the mean corpuscular volume (MCV) was 92 c.μ, the mean corpuscular haemoglobin (MCH) 32 $\gamma\gamma$, and the mean corpuscular haemoglobin concentration (MCHC) 35%. These estimations, which all gave normal values, were not repeated. The mean corpuscular diameter (MCD) on May 13, before operation, was 7.15 μ ±1,231, and on June 3, after operation, 7.34 μ ±0.626. Both mean diameters are within the normal range and do not differ significantly one from another (difference between means =0.19 μ: standard error of difference between means 0.138 μ). The large variation in red cell size, existing before operation, indicated by the S.D. of 1.231 μ, was due to the presence of both microspherocytes and macroreticulocytes, and this variation was significantly reduced after operation to an S.D. of 0.626 μ. The Price-Jones curves (Fig. 1) and the photomicrographs of the blood smears (Figs. 2 and 3) illustrate the change in the distribution of erythrocyte sizes after removal of the ovarian tumour.

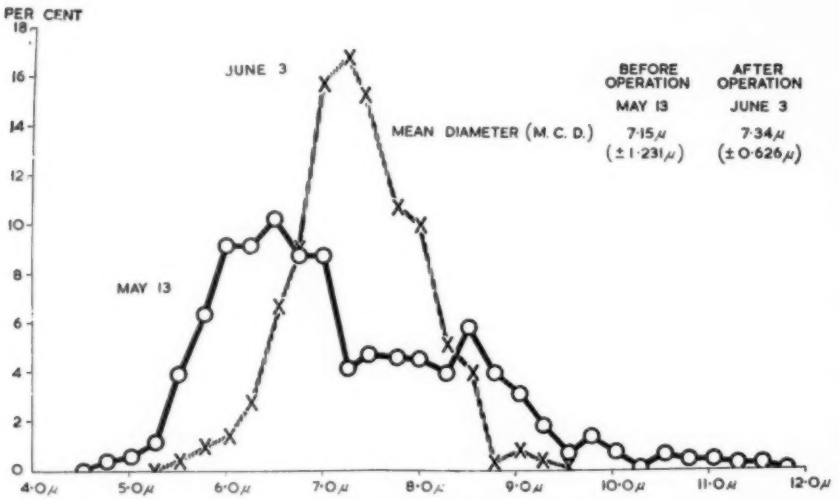

FIG. 1.—Frequency distribution of red cell diameters (Price-Jones curve). O——O abnormally spread curve during period of active haemolysis with peaks representing microspherocytes and macroreticulocytes. × - - - - × Normal curve 10 days after operation.

Microspherocytes.—These were a prominent feature of every blood film made before operation and were, in fact, the first clue in the diagnosis of haemolytic anaemia. These abnormal cells disappeared completely from the blood film two days after operation.

Nucleated Red Cells.—Nucleated red cells were seen in every blood film before operation and in none afterwards. On two occasions in the pre-operative course of the disease, they numbered 5 per 100 white blood cells. They were all late (orthochromatic) normoblasts.

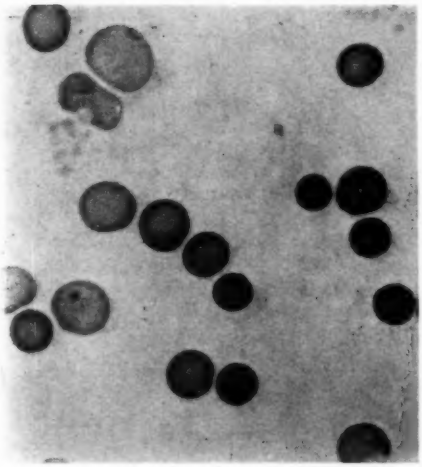

FIG. 2.—Blood film of May 13 showing microspherocytes and gross anisocytosis.

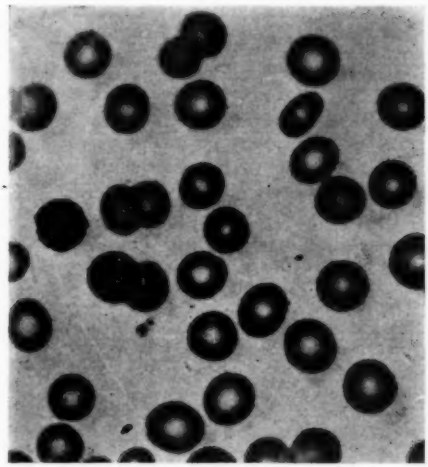

FIG. 3.—Blood film of June 3 showing normal red cells (a lymphocyte on left).

Reticulocytes.—The proportion varied between 33 and 47% of erythrocytes before operation, the highest count being made in the period of acute relapse. On the day after operation and after transfusion, reticulocytes were 10%, and fell to the normal figure of 0.3% four weeks later.

Leucocytes.—The total white cell count varied from 21,800 to 10,000 per c.mm. before operation, and from 15,400 to 8,400 per c.mm. after operation. There was no important abnormality of the differential leucocyte percentages.

Fragility of Red Blood Cells.—A greatly increased fragility in saline dilutions was noted on admission, some haemolysis even being evident in 0.9% saline. A month after operation the fragility curve was virtually normal (Fig. 4), and three months later lay well within normal limits with 50% haemolysis at 0.35% saline.

Cold Agglutinins.—These were not demonstrated in tests performed on May 9 and May 11 using the technique of Stats and Wasserman (1943).

Circulating Haemolysins.—No circulating haemolysins could be demonstrated at 4° C., 18° C., and 37° C. when the serum was tested against the patient's own washed corpuscles and the washed corpuscles of a healthy control subject of the same ABO group, with or without added guinea-pig complement.

The Donath-Landsteiner reaction for cold iso-haemolysins was negative.

The Race-Coombs test (developing test with anti-human-globulin rabbit serum) was negative on May 6.

Blood Groups.—Patient: Group O. Rhesus-positive. No atypical antibody.
Patient's mother: Group A. Rhesus-positive.

The Kahn test was negative.

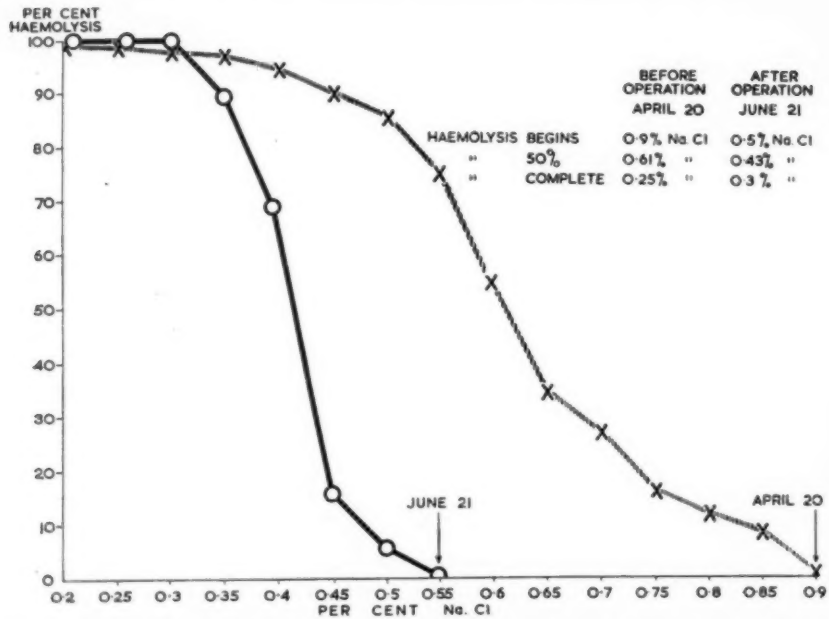

FIG. 4.—Erythrocyte fragility curves one month before and one month after removal of ovarian teratoma.

Faecal Urobilinogen.—This was estimated before and after operation.

Before operation—May	8	.. ..	600 mg./100 g. of fresh faeces
	17	.. ..	800 " " " "
	18	.. ..	520 " " " "
	19	.. ..	790 " " " "
After operation—June	9	.. ..	89 " " " "
	10	.. ..	79 " " " "

All the pre-operative values (determined by Maclagan's (1946) method : normal range 30–220 mg./100 g.) for faecal urobilinogen were greatly increased.

The test for faecal occult blood was negative.

Serum Bilirubin.—On April 21 the direct van den Bergh reaction was negative, and the indirect reaction was positive at 3.0 mg. bilirubin per 100 ml.

Serum Proteins.—On April 21 the total level was 6.5 g./100 ml. (albumin, 4.5 g./100 ml., globulin, 2.0 g./100 ml., A : G ratio, 2.25).

Sternal Marrow Puncture.—A puncture on May 16 gave the following results :

Total nucleated cells = 664,000 per c.mm.

Pro-erythroblasts	.. ..	2·8%	
Normoblasts A	.. ..	6·0%	62·8%
" B	.. ..	41·6%	
" C	.. ..	12·4%	

The results indicated a highly cellular bone marrow with a greatly exaggerated activity of red cell production, but no abnormality of other cellular elements except relatively decreased proportions.

The Ovarian Tumour

This rounded, smooth-surfaced tumour, measuring $7.5 \times 6 \times 4$ cm. in its main axes, weighed 100 g. Its thickened pedicle had been clamped and contained a portion of ovarian tissue. Fluid contents were aspirated through a wide bore needle and replaced by fixative. The tumour was opened after fixation and after the tooth and calcified tissue had been located on the *x*-ray screen.

The sliced tumour (Fig. 5) was seen to be a unilocular cystic teratoma filled with coils of hair and thick sebaceous matter and enclosed by a tough, smooth, fibrous wall. At a point on this wall a little removed from the pedicle there lay an eminence of solid

FIG. 5.—Unilocular cystic teratoma ("dermoid cyst") removed from right ovary on May 24.

tissue projecting 2 cm. into the cyst cavity. This mass contained the follicles from which most of the hair arose, and also the tooth, some bone, and many other tissues only identified microscopically. The tooth was unerupted.

Histology.—The thinner parts of the wall were formed of laminated collagenous fibrous tissue covered externally by peritoneum and lined within by much attenuated stratified squamous epithelium, beneath which were found a few skin appendages. The pedicle comprised a part of normal ovary, separated from the tumour by a band of fibrous tissue continuous with that in other parts of the cyst wall, but bearing larger and more prominent blood vessels. Sections prepared at varying distances through the solid eminence in the tumour showed an intermingling of tissues representing all three germinal layers and demonstrated that this, like every other neoplastic "dermoid" cyst, was a teratoma of tridermal origin. The following tissues in particular were noted: skin and skin appendages, a tooth and dental papilla, primitive myxoid connective tissue, adipose tissue, various types of fibrous tissue, trabeculated and compact bone, cartilage, gastric and intestinal mucosa. No splenic tissue was seen.

Cyst fluid, withdrawn before fixation, was turbid and opalescent. Centrifuging brought down a little amorphous deposit which contained no cells. The supernatant fluid remained milky. It contained large numbers of fat globules and smaller refractive lipoid particles but no identifiable cholesterol crystals. Serial dilutions of the supernatant fluid, from 1/5 to 1/2560, were made in saline and tested against washed human red cells of homologous group with and without added guinea-pig complement, but no haemolysis occurred in any tube of the test or of the controls at 18° or at 37° C.

Discussion

We have found only four earlier records, properly substantiated, of symptomatic haemolytic anaemia associated with ovarian tumours.

West-Watson and Young (1938) recorded such a case under the title of "Failed Splenectomy in Acholuric Jaundice." This was a woman of 44. The anaemia was extremely severe, red cells being less than 1.0 million per c.mm., nucleated red cells 36,746 per c.mm., and leucocytes 13,353 per c.mm. The spleen, which weighed 500 g., was removed, but the patient became worse. She was maintained by transfusions. Laparotomy, four months later in an attempt to find an accessory spleen, revealed no abnormality other than an ovarian tumour which was removed *faute de mieux*. Unexpectedly, the haemolysis ceased immediately and signs of toxaemia disappeared. The patient recovered completely. The ovarian tumour was a "dermoid cyst" in which no splenic tissue was found histologically.

Watson (1939) described the case of a girl aged 19 with two years' history of jaundice. Haemoglobin was 32%. The blood was moderately macrocytic, reticulocytes were 15%, saline fragility was normal. Faecal urobilinogen was greatly increased. A large ovarian cyst, containing 800 ml. of fluid-altered blood, was removed. There was no improvement in the anaemia. Pyrexial and haemolytic reactions followed transfusions. Finally the spleen, weighing 440 g., was removed. Another haemolytic crisis followed splenectomy, but thereafter the patient slowly recovered completely.

Singer and Dameshek (1941) reported a 47-year-old woman with severe anaemia, the blood showing spherocytosis, reticulocytosis, erythroblastosis, and increased saline fragility. Only temporary improvement followed splenectomy, but the patient was cured by the removal of a teratoma arising from the left ovary. This was a unilocular "dermoid" cyst 7 cm. in diameter. No definite splenic tissue was seen microscopically.

Jones and Tillman (1945) observed a woman aged 35 with severe anaemia (5–7 g. Hb/100 ml.), reticulocytes up to 20%, and slightly increased saline fragility. Her blood, which was Rh-negative, contained agglutinins to Group O Rh-positive cells and also to Group O and Group A Rh-negative cells. Auto-agglutinins were demonstrated, but cold agglutinins were not present. She also suffered from dermatomyositis, and had had attacks of intestinal obstruction. Obstructive peritoneal bands and a right ovarian cystic tumour were removed. The latter proved to be a pseudo-mucinous cystadenocarcinoma. The haemolytic anaemia appeared to have been relieved by this operation. There was a later acute intestinal obstruction.

Symptomatic haemolytic anaemia has been described in other morbid conditions. Carcinoma, sarcoma, leukaemia, and Hodgkin's disease are named by Watson, by Singer and Dameshek, and by Davis. Watson has also observed it in association with cirrhosis of the liver and with hyperthyroidism. Sarcoidosis and Gaucher's

disease are mentioned by Singer and Motulsky (1949), and acute infections by non-haemolytic micro-organisms by Singer and Dameshek. Fisher (1947) points to the frequency of increased haemolysis as a symptom in other blood diseases such as pernicious and related macrocytic anaemias, leuco-erythroblastic anaemia, and aplastic anaemia, but in these circumstances the criteria governing the definition of symptomatic haemolytic anaemia are not fulfilled.

The failure to demonstrate abnormal haemolysins, agglutinins, and atypical antibodies in our case in either the blood or the cyst contents is disappointing, since there is now much evidence to suggest that every variety of acquired haemolytic anaemia is due to some pathological haemolytic mechanism of this kind. Dameshek and Schwartz (1940) showed that spherocytosis is the morphological expression of an alteration in the structure of erythrocytes produced by various types of haemolytic substance, and microspherocytes were a prominent feature in the blood films of our patient. Loutit and Mollison (1946) demonstrated that red cells of patients with acquired acholuric jaundice on transfusion to normal recipients survive normally, whereas these same patients rapidly eliminated normal red cells transfused into them. They also showed that washed red cells of patients with acquired haemolytic jaundice were agglutinated by anti-human-serum rabbit serum, suggesting that these red cells had absorbed an immune antibody from their plasma. This latter observation was confirmed by Singer and Motulsky (1949), using anti-human-globulin rabbit serum as in the direct Race-Coombs test. The test was positive in seven cases of acquired haemolytic anaemia of unknown causation and in one case of symptomatic haemolytic anaemia associated with reticulo-sarcoma. It was negative in all except one gravely ill patient with haemolytic anaemia due to sulphonamide. Only one case of symptomatic haemolytic anaemia, that associated with reticulo-sarcoma, was so tested. In our patient the Race-Coombs test was negative, so we have no evidence to support, nor are we in a position to deny, the current theories of the mechanism of acquired haemolytic disease. One thing, however, is quite clear, that in such a case as ours the spleen does not play a dominant role. The haemolytic process must be conditioned by the presence of the tumour, and the conception of symptomatic haemolytic anaemia in these circumstances is fully justified.

There is a strong presumption that removal of the ovarian teratoma in this case cured the haemolytic anaemia, but this is not conclusively proved. Certain forms of acute haemolytic anaemia are arrested by blood transfusion. However, the relatively chronic course of the anaemia in this child, the absence of pyrexia at any time, the persistence of signs of haemolysis in the four days following the first transfusion, and the already known association of haemolytic anaemia with ovarian tumour are points which argue against transfusion being the effective therapy. West-Watson and Young specifically mention that transfusions in their patient failed to arrest the haemolytic process which, however, ceased immediately after removal of the ovarian teratoma. The possibility that our patient was, in fact, suffering from a crisis of familial acholuric jaundice can be dismissed because there was no earlier history to suggest the disease either in the patient or in her family ; after removal of the ovarian tumour red cell fragility returned completely to normal, and there were no further symptoms, in spite of the spleen being retained ; erythrocyte fragility tests, red and white cells and reticulocytes, and film appearances were all normal in the patient's mother, in her only sister, aged 7½, and in her only brother, aged 1 year.

Summary

A girl, aged 4 years 9 months, suffered from severe haemolytic anaemia characterized by microspherocytosis, reticulocytosis, greatly increased erythrocyte fragility, and an excessive output of faecal urobilinogen.

The haemolytic process was completely arrested following the excision of a unilocular cystic teratoma of the right ovary. The spleen, which was of normal size, was not removed.

Neither circulating haemolysins nor atypical agglutinins were demonstrated.

We are grateful to Mr. A. J. C. Latchmore for his surgical co-operation, to Professor F. S. Fowweather for the biochemical estimations, to Dr. H. S. Baar for his advice on the case, and to our technical assistants.

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A SIMPLE METHOD FOR DETECTING STREPTOMYCIN-RESISTANT TUBERCLE BACILLI

BY

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Since the advent of streptomycin therapy in tuberculosis, the need has arisen for a rapid, simple method for detecting the emergence of resistant strains of *Myco. tuberculosis*. Methods so far described, for example, that of the M.R.C. (1948), are mostly designed for accuracy, but they have the disadvantage of requiring considerable manipulation. The slide culture technique (Giammalvo, Natsios, and Elton, 1949) does give quick and apparently accurate results, but is of limited application when applied to pathological material, since large numbers of organisms must be present in the specimen. Beyond the recent work of Tinne and Henderson (1950) little attention seems to have been paid to diffusion methods such as the ditch plate. For these reasons a study has been made of the factors which affect the estimation of the sensitivity of *Myco. tuberculosis* to streptomycin, employing a diffusion gradient in Löwenstein's medium. As a result a simple and practicable method has been evolved, which is essentially a modification of that described by Tinne and Henderson.

Material and Methods

Source of Strains.—The strains of *Myco. tuberculosis* had all been recently isolated from patients in St. Thomas's Hospital and the King George V Sanatorium, Hydestile, Surrey.

Sensitivity Tests in Fluid Medium.—Except that fourfold instead of doubling dilutions of the antibiotic were used, all the strains used were tested for their sensitivity to streptomycin by the technique described by the Medical Research Council (1948).

Inocula.—Inocula were regularly taken from a 14-day-old culture in Dubos's fluid medium.

Preliminary Experiments

To discover whether Löwenstein's medium could be used in a diffusion method for determining the sensitivity of *Myco. tuberculosis* to streptomycin, experiments were made using ditch plates prepared as follows.

Amounts, each of 30 ml., of Löwenstein-Jensen medium were inspissated in 9-cm. Petri dishes at 80° C. for three hours. After drying, a ditch was cut on one side of the plate and filled with 2% agar containing streptomycin. Following an interval to allow the streptomycin to diffuse through the medium, the plates were inoculated by streaking the strains to be tested across the plate at right-angles to the ditch. The plates were then sealed with paraffin wax and incubated at 37° C. for three or four weeks to find

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(1) the optimum concentration of streptomycin to use in the "ditch," (2) whether or not diffusion occurs uniformly, (3) the optimum period to allow for diffusion to take place, (4) the effect, if any, imposed by time of incubation, (5) the effect of size of inoculum, and (6) methods of combating the high incidence of contamination which occurred.

(1) Plates containing ditches with six different concentrations of streptomycin (2,000, 750, 250, 125, 62, 31 γ/ml.) were made. The plates were immediately inoculated with six strains of *Myco. tuberculosis* chosen because they had been shown by the dilution method to have differing degrees of resistance to streptomycin.

Streaks of confluent growth became visible after 14 days. They were separated from the ditch by zones of inhibition of varying lengths, and all showed a sharp end-point.

The most satisfactory differentiation between sensitive and insensitive strains was obtained on plates containing 62 and 125 γ/ml. streptomycin in the ditch. For this reason, the concentration used in the ditch in all subsequent experiments was 100 γ/ml. streptomycin.

(2) Because Löwenstein's medium is made by inspissation, it is possible that the diffusion of a substance such as streptomycin might vary considerably from place to place in the same plate. This would seem improbable in view of the following experiment.

Löwenstein's medium, 35 ml., was inspissated in a 100-ml. screw-capped medical flat bottle laid on its side. Condensation water was removed with a sterile pipette, and the bottle laid on a horizontal surface with the medium in the vertical plane. Then 10 ml. of 2% agar containing 100 γ/ml. streptomycin was run in and allowed to set. A 14-day culture of the strain H 37 in Dubos's fluid medium was diluted 1:10 in sterile normal saline and 1 ml. flooded over the surface of the medium. Even distribution of inoculum was ensured by rocking the bottle. The bottle was turned on its face and excess fluid removed with a pipette.

After incubation for four weeks, growth had taken place up to a straight line running parallel with the edge of the streptomycin containing agar, indicating that there had been uniform diffusion of streptomycin through the medium.

(3) To determine the optimum time to allow for diffusion to take place, six plates were made from a single batch of medium. They were divided into three pairs, the ditches of which were filled five days, two days, and immediately before inoculation. During the period allowed for diffusion to take place, the plates were stored at room temperature.

TABLE I

RELATION OF TIME FOR DIFFUSION TO ZONE OF INHIBITION

Diffusion Time (in days):		0	2	5	
Strain ..	H 37	9	18	21	Zone of inhibition (in mm.)
	711	4	8	10	
	681	0	0	0	

After incubation the zones of inhibition were measured (Table I), and it is obvious that better differentiation was obtained by allowing a period for diffusion of streptomycin to take place before inoculation. For this reason, plates have been kept for three days at bench temperature.

(4) Having established that satisfactory results could be obtained by using 100 γ/ml. streptomycin in the ditch and by allowing a period of three days for diffusion to take place, experiments were made to see if the zone of inhibition decreased in length as

incubation was prolonged. While this did occur, the zones of inhibition measured at two, three, and four weeks tending to recede by amounts varying from 0 to 4 mm., in no case did the essential pattern change.

(5) Because the apparent resistance to streptomycin of *Myco. tuberculosis* is known to increase with the size of the inoculum (Smith and Waksman, 1947) experiments were made to determine the extent to which the size of inoculum affects the results obtained using ditch plates. For this purpose, serial fivefold dilutions of fluid cultures of seven strains were made, and a standard loopful of each dilution was streaked across the plate at right-angles to the ditch and the zones of inhibition measured after three weeks. The results obtained are shown in Table II.

TABLE II

RELATION OF INOCULUM SIZE TO ZONE OF INHIBITION (MM.) WITH AND WITHOUT 100 U./ML. CRYSTALLINE PENICILLIN G IN THE MEDIUM

		Löwenstein's Medium							
Dilution of Inoculum:		1:1	1:5	1:25	1:125	1:625	1:3125	1:15625	1:78125
Strain	12C	20	20	22	24	26	26	30	—
	33C	0	0	0	0	0	0	10	0
	45C	20	23	25	25	25	26	26	27
	47C	17	20	22	23	23	25	25	30
	55C	10	11	11	Contaminated				
	80C	0	9	15	15	23	—	—	—
	20C	23	24	24	28	28	31	31	—

		Löwenstein's Medium plus 100 u./ml. Penicillin							
Dilution of Inoculum:		1:1	1:5	1:25	1:125	1:625	1:3125	1:15625	1:78125
Strain	12C	19	23	24	24	26	30	27	—
	33C	0	0	0	0	5	0	0	0
	45C	18	19	21	22	23	25	26	26
	47C	16	19	21	22	23	23	23	24
	55C	11	12	14	15	16	16	16	17
	80C	0	7	11	18	24	32	32	35
	20C	23	23	25	26	27	27	29	—

It is evident that when a strain is highly resistant the size of inoculum is immaterial, but with strains that are less resistant there is a tendency for the zone of inhibition to lengthen as the inoculum is decreased With some resistant strains (80C), however, this effect is particularly marked. It is therefore clear that the size of inoculum should be controlled as far as possible. Examination of 40 consecutive recently isolated strains of varying degrees of sensitivity confirmed these findings.

(6) There was a high incidence of contamination in the above experiments due to the amount of manipulation required. This was in part reduced by replacement of the original lids at the time of inoculation with dry, sterile lids, and partly by inspissating the plates with a "false ditch" made of 4% agar. This is easily removed and quickly replaced by the ditch containing streptomycin when the medium has solidified. It was also found that if crystalline penicillin G at a concentration of 100 u./ml. were incorporated in the medium, contamination was virtually abolished but with no apparent alteration in the sensitivity of the strains. The results also given in Table II were obtained

with plates which were treated in exactly the same way as those in the upper half of the Table but which contained 100 u./ml. penicillin.

Thus, although ditch plates give satisfactory results, they possess certain obvious disadvantages in that they involve much preparation and are extravagant of medium. A simple method was therefore evolved in which the vertical diffusion principle of Tinne and Henderson (1950) was adopted.

A Diffusion Method Using 1-oz. Bottles

Amounts of Löwenstein's medium, each of 5 ml., are inspissated in 1-oz. screw-capped bottles which are laid flat so that a reasonably constant depth of medium is achieved. Condensation water is removed with a sterile pipette. Then 1 ml. of 2% agar containing 100 γ/ml. streptomycin is introduced and allowed to set on the bottom of the bottle as shown in Fig. 1. From this point forward, the bottle is kept vertical and any condensation water which may develop collects at point A between the wall of the bottle and the agar and does not interfere with the reading of the results. Inoculation is made by running one drop of a 14-day culture in Dubos's fluid medium of average turbidity from a "50 dropper" pipette down the surface of the medium. It is not difficult to inoculate each bottle with an unknown and a control strain. When a direct test is made using pathological material, the concentrate obtained by using Petroff's method is inoculated in two dilutions, 1:1 and 1:10. In this way a rough indication of the bacterial density of the inoculum is obtained.

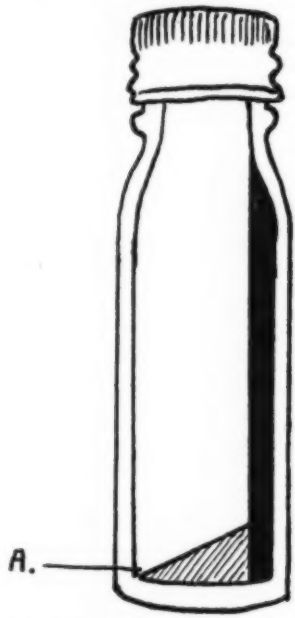

FIG. 1.—Drawing to show the method of using 1-oz. bottles for detecting strains of *Myco. tuberculosis* resistant to streptomycin. Condensation water collects at point A. Löwenstein's medium = black. Streptomycin "ditch" shaded.

Whilst it is clear that most of the points covered in the preliminary experiments are applicable to the method just described, certain details were further investigated using 1-oz. bottles.

To find the best time to inoculate the "diffusion bottles" after adding the streptomycin, streptomycin agar was added to bottles containing Löwenstein's medium on five consecutive days. On the fifth day all the bottles were inoculated with one drop of a 14-day culture of a sensitive strain of *Myco tuberculosis* in Dubos's fluid medium.

It was found that the length of the zones of inhibition were most regular and the end-points obtained were most distinct where five days had elapsed between the introduction of streptomycin and the time of inoculation. However, satisfactory results were obtained if the bottles were inoculated immediately after adding the streptomycin, and it has been found more convenient to follow this practice.

Because it was thought that the diffusion of streptomycin might be influenced by the slope of the "ditch," 15 bottles were made whose ditches were allowed to set with the long axes of the bottles making various angles with the horizontal plane. All were inoculated in the same way. The results observed indicated that any variation in the rate of diffusion of streptomycin due to the angle of the ditch is not sufficient to interfere with results.

Comparison of Results Obtained by the Dilution and Diffusion Bottle Methods

Parallel determinations of the sensitivity to streptomycin of 49 strains of *Myco. tuberculosis* were made using the M.R.C. dilution method and diffusion bottles. In every case the same inoculating culture was used for both methods. All the inoculating cultures were of similar turbidity, and the volume applied to each bottle

TABLE III

COMPARISON OF THE RESULTS OF PARALLEL DETERMINATIONS OF STREPTOMYCIN SENSITIVITY OF 49 STRAINS USING DILUTION AND DIFFUSION METHODS

Length of Zone of Inhibition Relative to that of H 37 in Diffusion Bottles	Resistance Relative to H 37 by Dilution Method					Total No. of Strains
	<1	4	16	67	256<	
0	1	—	1	—	7	9
<¼	—	—	1	3	5	9
¼–½	—	1	—	1	1	3
½–¾	4	1	—	—	—	5
¾–1	16	2	—	1	—	19
1<	4	—	—	—	—	4
Total no. of strains	25	4	2	5	13	49

was controlled by using the same dropping pipette for both methods. The results are given in Table III relative to those derived from H 37. In the case of the diffusion method the ratio

$$\frac{\text{zone of inhibition of test strain}}{\text{zone of inhibition of H 37}}$$

is employed to record the sensitivity of the strain under test. Whilst this is a convenient form in which to record results, it must not be inferred that there necessarily exists any simple relationship with the ratio

$$\frac{\text{minimum concentration streptomycin inhibiting test strain}}{\text{minimum concentration streptomycin inhibiting H 37}}$$

which is used to express the results derived from the dilution method. Nevertheless, it is clear that in most cases the results obtained by both methods agree. In two instances, however, the results obtained by the two methods were at variance, one strain being apparently resistant to streptomycin by the diffusion method and sensitive by the dilution method, the other strain behaving conversely. These two estimations have not been repeated, and no explanation can therefore be given for these discrepancies.

Discussion

Using diffusion gradients of streptomycin in Löwenstein's medium, two methods for determining the resistance of *Myco. tuberculosis* have been evolved.

The method using 1-oz. bottles is simple and rapid. It gives a result when applied directly to pathological material in about four weeks and can be carried out in any small laboratory. Secondly, because it may be applied directly to pathological material, it is likely to give a more faithful representation of the resistance to streptomycin of the organisms in the host than methods in which one or more

subcultures have to be made. Both these advantages are shared by the method described by Holt and Cruickshank (1949) in which parallel cultures are made on a number of Löwenstein slopes containing different concentrations of streptomycin.

Nevertheless, there are a number of disadvantages. The method is, at best, an approximate one. But this is little handicap, because the important consideration is whether the strain in question has, or has not, developed sufficient resistance for streptomycin treatment to be stopped. A more important disadvantage lies in the necessity for controlling the size of the inoculum. Tinne and Henderson (1950) make no mention of attempting to control this factor, apart from using a standard loop, but something more than this is required. Although the error entailed by neglecting this factor is usually not great if the test is regarded as approximate, there are certain strains in which the result is markedly affected by the size of the inoculum. With these strains, it is important to know the results obtained with both large and small inoculum.

Quite another use can be made of the diffusion gradient in the form of a streptomycin ditch in a Löwenstein plate. Here all the advantages inherent in ditch-plate techniques are available.

In the course of the above experiments three strains were found in which the apparent sensitivity was dependent upon the size of inoculum. With all three strains the large inocula grew up to the ditch containing 100 γ/ml. streptomycin, but the small inocula showed zones of inhibition of from 20 to 30 mm. Behaviour of this kind is similar to that obtained on penicillin ditch plates with staphylococci which produce penicillinase (Barber, 1947). Whereas it is possible that these strains produce a substance antagonistic to streptomycin, the same picture might be obtained with either a mixture of sensitive and resistant strains or with an exaggeration of the variation in sensitivity to streptomycin on the part of individual cells described by Pyle (1947). These three strains are being further investigated with these points in mind.

In conclusion, it appears that methods employing diffusion gradients of streptomycin in Löwenstein's medium have many applications both as a simple method of detecting the emergence of resistance to streptomycin in *Myco. tuberculosis* and in the more detailed study of the behaviour of resistant strains.

Summary

An account is given of various factors affecting estimations of the sensitivity of *Myco. tuberculosis* to streptomycin by means of diffusion techniques.

A simple method which can be applied directly to pathological material is described.

My thanks are due to Professor Ronald Hare for his helpful criticism and to Mrs. D. N. Twort for technical assistance.

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A LATE MANNITOL-FERMENTING STRAIN AND RAPIDLY FERMENTING VARIANT OF *SHIGELLA FLEXNERI* TYPE Z

BY

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Shigella organisms are divided biochemically into two main groups distinguished by their ability to ferment mannitol. Non-mannitol-fermenting strains of *Sh. flexneri* type 88 (biochemical type *newcastle*) and of *Sh. flexneri* type 103 (Mumford and Mohr, 1944) are, however, well recognized, and it is probable that similar variants occur among other mannitol-fermenting dysentery bacilli.

The organism described in this paper is of interest not only as a late-mannitol-fermenting variant of *Sh. flexneri* type Z, but also because the strain itself gave rise to variants which fermented mannitol within 24 hours. The strain was originally isolated by Dr. Rickards at the Royal Lancaster Infirmary from a patient with a typical bacillary exudate. Dr. Rickards found that the organism was agglutinated by Flexner Z serum, though it failed to ferment mannitol. The culture was sent to the Dysentery Reference Laboratory, where its identification was confirmed serologically as *Sh. flexneri* type Z.

Behaviour of Parent and Variant Strain

On heart agar and MacConkey's medium the strain produced colonies whose appearance and smell were characteristic of *Sh. flexneri*. It was non-motile, indole-positive, and H_2S-negative. Acid was produced from glucose after 24 hours, but not from maltose, lactose, sucrose, salicin, or dulcitol after 14 days' incubation. Mannitol was fermented late, usually about the fifth day.

In view of the results obtained with *Sh. sonnei* and a number of late-fermenting paracolon bacilli (Cook, Knox, and Tomlinson, 1951), it was thought probable that the slow fermentation of mannitol by this strain was associated with the production of biochemical variants capable of rapid mannitol utilization. In an attempt to isolate these variants the parent culture was plated on to an agar medium containing mannitol and neutral red. After three or four days' incubation at 37° C. both single colonies and the area of confluent growth showed a number of fermenting papillae from which a pure growth of a rapid mannitol-fermenting strain was readily obtained. The biochemical and serological behaviour of this variant strain was identical with that of the parent culture, except for the ability of the variant to produce acid from mannitol within 24 hours both on a solid medium and in peptone water. The rapidly fermenting variant bred true over a period of many months on agar not containing mannitol and still retained its power of rapid mannitol fermentation.

The time-relation between the appearance of variant cells and fermentation of mannitol in a liquid culture was shown by the following experiment. The parent or late-fermenting culture was grown in 1% mannitol peptone water containing Andrade's indicator, and daily subcultures were made on to mannitol-agar plates containing neutral red. Rapidly fermenting colonies were first detected on sub-

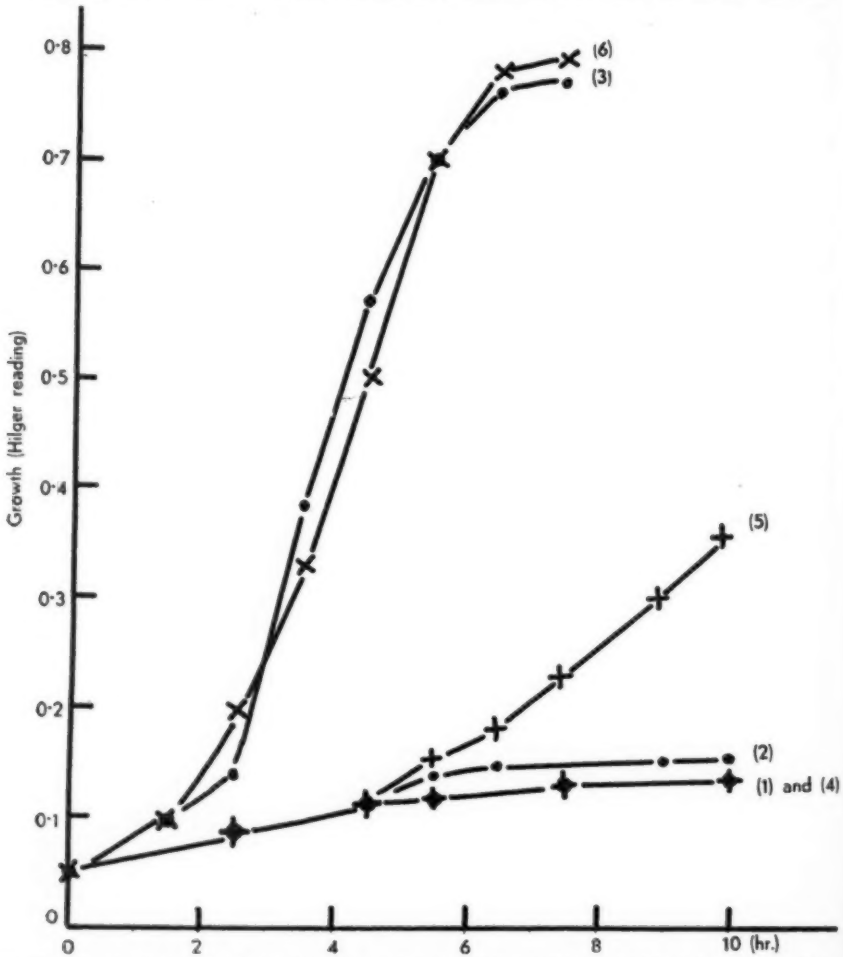

FIG. 1.—Graph of growth of parent culture and mannitol-fermenting variant in buffered mannitol and glucose peptone water and in buffered peptone water. (1) Parent culture grown in buffered peptone water. (2) Parent culture grown in buffered mannitol peptone water. (3) Parent culture grown in buffered glucose peptone water. (4) Mannitol variant grown in buffered peptone water. (5) Mannitol variant grown in buffered mannitol peptone water. (6) Mannitol variant grown in buffered glucose peptone water.

culture after two days' incubation of the peptone water ; on subculture the following day these had increased to approximately half the total number of colonies on the plate. No change of *p*H, however, was observed in the mannitol peptone water until the fifth day, by which time the fermenting colonies formed approximately 80% of the total colonies obtained on subculture.

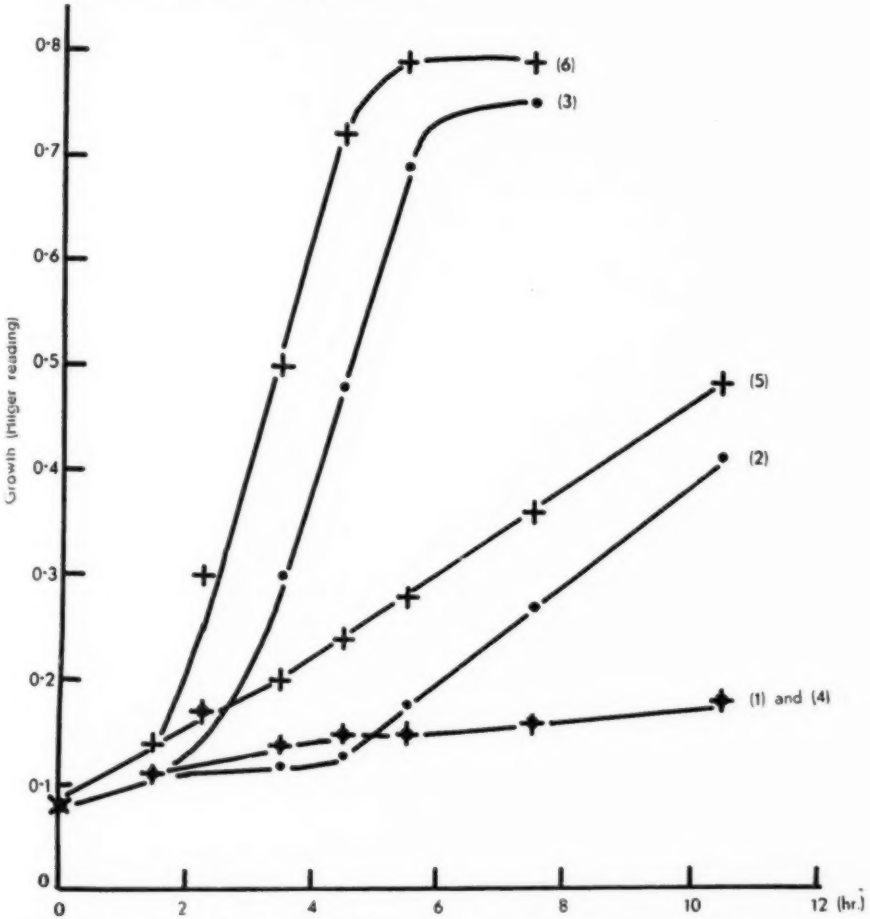

FIG. 2.—Graph of growth of mannitol-fermenting variant in buffered mannitol and glucose peptone water after subculture on agar with and without mannitol. (1) Subcultured from agar and grown in buffered peptone water. (2) Subcultured from agar and grown in buffered mannitol peptone water. (3) Subcultured from agar and grown in buffered glucose peptone water. (4) Subcultured from mannitol agar and grown in buffered peptone water. (5) Subcultured from mannitol agar and grown in buffered mannitol peptone water. (6) Subcultured from mannitol agar and grown in buffered glucose peptone water.

The ability of the parent and variant cells to utilize mannitol was investigated by comparing the growth rates of each culture in buffered peptone water with 1% mannitol, in peptone water with 1% glucose, and in peptone water alone. The growth rates were measured by recording the turbidity readings in a "Hilger biochem" absorptiometer. Both cultures grew well in the presence of glucose and poorly in the basal medium without additional carbohydrate. The presence of mannitol conferred no advantage to the parent culture, but improved the growth of the variant cells after a delay of four or five hours (Fig. 1). These results suggested that the variant cells in a growing culture were able to utilize mannitol only after a period of enzymatic adaptation. Evidence supporting this was obtained by comparing the growth rates of the variant strain after subculture on media with and without mannitol (Fig. 2). Previous contact with the substrate almost completely abolished the "lag period" of several hours observed in mannitol fermentation, although it did not increase the rate of growth. Previous contact with mannitol also reduced the short lag period observed in glucose fermentation, though this reduction was not so marked as in the case of mannitol.

Discussion

The isolation from a case of bacillary dysentery of a strain of *Sh. flexneri* type Z which failed to ferment mannitol in 24 hours emphasizes once again the need for constant vigilance in the routine investigation of intestinal organisms (Nelson, 1947). Rapid mannitol-fermenting variants were obtained from this strain, and the behaviour of the parent and variant cultures proved of some interest in relation to the mechanism of delayed fermentation.

In the late fermentation of sucrose by *Sh. sonnei* Cook and his colleagues (1951) have demonstrated two distinct processes, viz. mutation, a hereditary change independent of the substrate, and substrate-induced adaptation: it would appear that both these processes are also involved in the fermentation of mannitol by the strain of *Sh. flexneri* described in this paper. The variant cells produced by this strain, having permanently acquired the property of rapid mannitol fermentation and being able to transmit this characteristic to succeeding generations in the absence of the substrate, can probably be regarded as true mutants of the parent culture. These cells were present in relatively small numbers during the early stages of growth of the parent culture in mannitol peptone water, though their proportion steadily increased until the substrate was fermented, the mannitol probably acting as a selective agent. This association of the production of rapidly fermenting mutant cells with delayed fermentation has been observed by a number of other workers (Lewis, 1934 ; Sears and Schoolnik, 1936 ; Cook *et al.*, 1951). Growth experiments with the variant culture showed that some degree of enzymatic adaptation occurred before the cells were able to utilize mannitol.

There is one difference, however, between this fermentative variant and those produced by *Bact. coli mutabile*, *Sh. sonnei*, and certain paracolon bacilli. The biochemical reactions of these parent cultures are typical of the species or group, while those of the variant cultures are abnormal. In the case of the mannitol-fermenting variant of *Sh. flexneri* the parent cells are themselves atypical in fermenting mannitol late, and it is in fact the variant culture whose biochemical reactions are characteristic of the species.

Summary

A late mannitol-fermenting strain of *Sh. flexneri* type Z causing rapid mannitol-fermenting variants is described. Rapid fermentation during growth of the variant was a stable characteristic unchanged by subculture in the absence of mannitol. It is concluded that in the late fermentation of mannitol by the parent strain two distinct processes, namely mutation and substrate-induced adaptation, were concerned.

My thanks are due to Colonel R. F. Bridges, Dysentery Reference Laboratory, Oxford, for carrying out the serological tests on both the parent and variant strains.

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THE DETERMINATION OF PROPYLTHIOURACIL IN URINE WITH 2:6-DICHLOROQUINONE-CHLOROIMIDE

BY

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Previous colorimetric methods for the determination of antithyroid compounds of the thiouracil type have been based upon the bluish-green colour which is given when compounds containing the C=S group react with Grote's (1931) reagent. Methods for the determination of thiouracil levels in urine using this reaction have been described by Anderson (1944), Williams, Jandorf, and Kay (1944), Chesley (1944), Christensen (1945), Paschkis, Cantarow, Rakoff, and Tillson (1945), and others. With the exception of Williams *et al.* (1944), all of these workers have reported on the instability of the stock Grote reagent, which necessitates not only the frequent preparation of fresh reagent, but also the inclusion of standards with each set of unknowns. The method of Williams and his colleagues, however, has the disadvantage of a 12-hour digestion procedure and the inconvenient adjustment of the reaction with a strong base.

Recently Olson, Ely, and Reineke (1947) have claimed to have produced a stable Grote reagent by the application of a suitable ageing process.

Since the method described here for the colorimetric determination of propylthiouracil involves a new colour reaction, it is not necessary to review further previous work.

A new colour reaction for antithyroid drugs of the thiouracil type has already been described (McAllister, 1950). This is based upon the reaction of the compound in a buffer at *p*H 8.0 with 2:6-dichloroquinone-chloroimide to give a yellow chloroform-soluble complex. This colour reaction has also been shown to be specific for the newer antithyroid compounds of the mercaptoimidazole type (McAllister, 1951a), and has been applied on a quantitative basis to the determination of methylthiouracil in urine (McAllister, 1951b). In applying this colour reaction to the determination of propylthiouracil in urine, certain improvements have been introduced, the most important of these being a preliminary ether extraction of the compound from the urine at *p*H 6.0.

Experimental

Gibbs (1927a, b) has shown that 2:6-dichloroquinone-chloroimide reacts with phenols, unsubstituted in the para-position, with the formation of blue or violet

indophenol pigments. The reagent has also been used for the detection of thiamine by Raybin (1938) and by Scudi (1941) for the estimation of pyridoxin. Fearon (1944) has described a method for the determination of uric acid in urine which is based upon the yellow, the intensity of which is given when this compound reacts with the chloroimide reagent at *p*H 9.2. In this paper Fearon also noted the behaviour of various amino compounds and urinary solutes in the reaction at this *p*H.

The colour reaction for other compounds has been found to be dependent upon the *p*H of the reaction mixture ; it is more dependent indeed than the reaction for the thiouracils, and by using a *p*H of 8.0 the reaction with most amino-acids is considerably slowed down. Furthermore, uric acid, the most reactive urinary constituent in the test, gives a slower reaction with the reagent at this *p*H.

Scope of the Chloroimide Reaction.—Solutions of the various compounds listed in Table I were dissolved in distilled water, and where necessary the *p*H of their solutions raised to 8.0. Five ml. of buffer-chloride solution, *p*H 8.0, was then added, followed by 0.1 ml. of a 0.4% solution of 2 : 6-dichloroquinone-chloroimide in aldehyde-free absolute ethanol. Colour reactions were then allowed to proceed for one hour at room temperature.

Five ml. of chloroform was then added to each and the mixtures vigorously shaken. Results are given in Table I.

Results

With the exception of thiourea, none of the compounds tested gave colorations which were removable from the aqueous phase by means of chloroform.

Uric acid was the most reactive of the group tested. At *p*H 8.0 the colour given by this compound tends to be orange, whereas at higher *p*H values it is pure yellow.

The results obtained with the amino-acids in the test were in agreement with those reported by Fearon (1944), although his reactions were carried out at *p*H 9.2. Amino compounds in general react very slowly with the chloroimide reagent and give colorations which are weak in comparison to the amount (2 mg.) under test.

Colour reactions with the chloroimide reagent are very dependent upon the *p*H of the reaction mixture. At *p*H values below 8.0, practically no colorations are given by amino-acids, and the reaction with other compounds is slowed down considerably. At *p*H values of 10 and above, the reagent tends to undergo a spontaneous decomposition, with the formation of a reddish mixture of pigments. Gibbs (1927a, b) found that this reagent decomposition was accelerated by exposure to light, and Fearon found that it could be repressed by the inclusion of sodium chloride in the buffer mixture. In this work, due mainly to the lower *p*H value used, reagent decomposition has not been encountered.

Fearon (1944) has shown that the colour reaction for thiourea is catalysed by copper. Although the colour reaction for this compound is rendered specific by removing the complex from the aqueous phase by means of chloroform, even with catalysis of the reaction with the metal it is not sensitive enough to be used on a quantitative basis. Fearon has also shown that the colour given by uric acid in the reaction is turned red by the addition of silver salts. In this work no such catalysis of the colour reaction for the thiouracils by metals has been observed, nor any silver binding NH group effect.

TABLE I

COLOUR REACTIONS AT *p*H 8·0 OF VARIOUS COMPOUNDS WITH 2:6-DICHLOROQUINONE-CHLOROIMIDE

Compound (2 mg. of each or amount noted)	Colour Reaction	Solubility of Coloured Complex in Chloroform
Pyrimidines		
Uracil	No reaction	—
Thymine	" "	—
Purines		
Uric acid	Rapid orange	Insoluble
Guanine HCl	No reaction	—
Adenine HCl	" "	—
Xanthine	Rapid yellow	Insoluble
Amino Compounds		
Ammonia	No reaction	—
Methylamine	Slow violet	Insoluble
Glycine	" "	"
Alanine	Faint pink	"
Serine	" "	"
Valine	No reaction	—
Cystine	" "	—
Tyrosine	" "	—
Tryptophane	" "	—
Histidine	Slow; faint violet	Insoluble
Arginine	No reaction	—
Histamine	" "	—
Water-soluble Vitamins		
Ascorbic acid	No reaction	—
Aneurin	Yellow, turns brown	Insoluble
Pantothenic acid	No reaction	—
Pyridoxin	Blue, decomposes	Insoluble
Riboflavin	No reaction	—
p-Amino-benzoic acid	" "	—
Biotin (100 μg.)	" "	—
Urinary Solutes		
Urea	No reaction	—
Creatine	" "	—
Creatinine	Faint pink	Insoluble
Allantoin	No reaction	—
Hippuric acid	" "	—
Miscellaneous		
Alloxan	No reaction	—
Dialuric acid	Rapid yellow	Insoluble
Barbituric acid	Rapid violet	"
Barbitones	No reaction	—
Sulphonamides	" "	—
Glucose	" "	—
Lactose	" "	—
Fructose	" "	—
Thiourea	Slow violet	Soluble
Methylamine	" "	Insoluble

The Colour Reaction for Propylthiouracil

The colour reaction for 2-thiouracil and its 4-methyl derivative has already been described (McAllister, 1950, 1951b). A similar reaction is given by 6-n-propylthiouracil, but the yellow product is more soluble in chloroform.

Reagents.—The following reagents were used in the test :

Solution of 0.4% 2 : 6-Dichloroquinone-chloroimide.—A 0.4% solution of the compound in aldehyde-free absolute ethanol is stored in a brown bottle ; the reagent will keep for about six weeks.

Buffer-Chloride Solution pH 8.0.—To 50 ml. of a 0.2 M. solution of boric acid in 0.2 M. potassium chloride was added 3.97 ml. of 0.2N. sodium hydroxide solution. Then 100 ml. of a 20% aqueous solution of pure sodium chloride was added, and the volume of the mixture made up to 200 ml. with distilled water.

Since the sodium chloride depresses the *p*H of the mixture the *p*H should be checked and then adjusted to 8.0 by the addition of decinormal soda.

Standard Propylthiouracil Solution.—This was prepared by dissolving 25 mg. of 6-n-propylthiouracil in 5 ml. of aldehyde-free absolute ethanol and then making the volume to 250 ml. with distilled water. The solution will keep for about one week. It contains 100 μg. per ml.

Procedures.—The following procedures were used in the development of the colour reaction :

Chloroform/Water Partition of the Coloured Complex.—To 1 ml. (100 μg.) of the standard propylthiouracil solution was added 4 ml. of water and 5 ml. of the borate buffer at *p*H 8.0. Then 0.1 ml. of the 0.4% chloroimide reagent was added, and the colour allowed to develop for 45 minutes. Then 10 ml. of chloroform was added to the mixture and the tube well shaken. The distribution of the coloured complex in the solvent and aqueous layers was examined in this and in another series containing 200 μg. of propylthiouracil treated similarly.

For purposes of comparison, the results obtained with thiouracil and methylthiouracil treated likewise are also included (Table II).

TABLE II

CHLOROFORM/WATER PARTITION OF COLOURED COMPLEXES AT *p*H 8·0

Compound (100 μg. each)	Colour Reaction	Approximate Percentage of Colour Distributed after Shaking*	
		Aqueous (10 ml.)	Chloroform (10 ml.)
2-Thiouracil	Yellow	50	50
Methylthiouracil	,,	—	100
Propylthiouracil	,,	—	100

* A similarly treated series containing 200 μg. of each of the above compounds gave : thiouracil, approximately 25% removed by 10 ml. solvent; methylthiouracil, approximately 50% removed by 10 ml. solvent; propylthiouracil, 100% removed by 10 ml. solvent.

Results

As will be seen from the results given in Table II considerable differences in the solubility of the reaction products are exhibited. Absorptiometer measurements of the colours given by equal weights of thiouracil, methylthiouracil, and propyl-

thiouracil were found to be approximately the same. This would appear to show that the type of substituent on the thiouracil ring does not influence the colour reaction, but does have a marked effect on the solubility of the coloured reaction products in chloroform.

In previous work it was found that the colour reaction for the thiouracils could be accelerated by shaking during the development period, but once the latter had been completed this effect was vitiated. A similar aeration effect has been noted by Fearon (1944) in the chloroimide reaction for glycine, thiourea, and indole.

Effect of *p*H on the Solubility of the Coloured Complexes.—The *p*H of the reaction mixture has a marked effect on the solubility of the coloured products in the solvent. As the *p*H rises, the solubility of the coloured products in chloroform decreases, until at *p*H 10 none of the colour given by 100-μg. amounts of thiouracil and methylthiouracil is removable from the aqueous phase by means of the solvent. Propylthiouracil, on the other hand, gives, in amounts up to 100 μg., colorations which can be completely removed from the reaction mixture at *p*H 10 by 10 ml. chloroform.

Quantitative Aspects of the Colour Reaction.—This has already been reported for thiouracil and its 4-methyl derivative (McAllister, 1951b). For propylthiouracil the sensitivity of the reaction is about 20 μg. In the aqueous phase, the colour has been found to be stable for at least 60 minutes, and in chloroform for at least 30 minutes, as determined by absorptiometer measurements.

The colour system obeys Beer's law with concentrations of propylthiouracil up to 150 μg., and the graphs obtained by plotting concentrations against absorptiometer readings are reproducible. Amounts of propylthiouracil in excess of 150 μg. give colours which can be diluted with further amounts of chloroform.

Determination of Propylthiouracil in Urine

When the colour reaction is applied direct to dilute samples of urine satisfactory recovery values can be obtained in the case of methylthiouracil (McAllister, 1951b). Further experience of this method, when applied to the determination of propylthiouracil in urine, has shown that when the urinary level of the drug is low there is a tendency for the reagent to be taken up by other urinary solutes before the drug reaction can be completed. This may be overcome to a certain extent by the use of a more concentrated reagent—for example, 0.8%—but under such conditions the colours given by the higher amounts of propylthiouracil (200 μg.) are partially destroyed by aeration during the extraction process. During attempts to overcome this difficulty it was found that propylthiouracil could be quantitatively removed from urine at *p*H 6.0 by shaking with ether.

Ether Extraction of Propylthiouracil.—The reagents which were employed for the colour reaction have already been described. Anaesthetic ether was employed for the extraction.

Procedure.—Complete 24-hour samples of the urine were collected in bottles containing 10 ml. of chloroform. Analysis had to be carried out without delay.

Urine, 100 ml., was measured out and the *p*H adjusted to 6.0 by the usual methods. Of this, 50 ml., or an aliquot containing up to 5 mg. of propylthiouracil, should be taken, and transferred to a small separating funnel. The urine was then extracted three times

with 100-ml. amounts of peroxide-free ether. The ether extracts were then pooled and washed once with 100 ml. of distilled water. The combined ether extracts were then taken to dryness on a steam bath. To the dry residue was then added 2 ml. of aldehyde-free absolute ethanol, and the mixture shaken until solution was effected. The solution was then washed into a 100-ml. volumetric flask and made to the mark with distilled water.

Development of Colour.—Aliquots, of 2 ml. and 1 ml., were taken and the volume of each adjusted to 5 ml. with water. Then 5 ml. of the borate-buffer, *p*H 8.0, was added to each, followed by 0.1 ml. of the 0.4% chloroimide reagent. After mixing, the tubes were allowed to stand for 45 minutes, when 10 ml. of chloroform was added to each, and the mixtures well shaken until all of the yellow had been extracted by the solvent. The chloroform extracts were allowed to settle, and the aqueous supernatant in each removed by suction. They were then filtered through a small No. 42 Whatman filter paper, and read in a Spekker absorptiometer, readings being taken against chloroform and using the Spekker violet filter.

Standard Reference Graph.—The colour reaction which has been described was applied to 10, 20, 30, 40, 50, and 100 μg. of propylthiouracil each in a volume of 5 ml. water, and the colours given by each amount extracted with 10 ml. chloroform. No colour was given by the reagent blank.

Results

Recovery at Various *p*H Values.—Various amounts of propylthiouracil dissolved in aldehyde-free absolute ethanol were added to normal samples of urine. Aliquots, 100 ml., of each of these were taken, and the *p*H of each adjusted to 6.0, 7.0, and 8.0 by the usual methods. Then 50-ml. aliquots of each were removed and extracted with three 100-ml. amounts of ether. The propylthiouracil content of each was determined by the method which has been described. Results are given in Table III.

TABLE III

RECOVERY OF PROPYLTHIOURACIL FROM URINE AT VARIOUS *p*H VALUES

Urine *p*H	Propylthiouracil Added (mg.%)	Propylthiouracil Found (mg.%)	Recovery (%)
1. 6·0	10·0	8·49	84·9
2. 7·0	10·0	6·43	64·3
3. 8·0	10·0	5·30	53·0

The best recovery values were obtained with urine at *p*H 6.0. Thiouracil types of compounds are readily broken down at *p*H 8–9, and this may account for the low values obtained at *p*H 8.0, although some other factor may be involved.

Material at Ether/Water Interface.—The ether extraction of urine often results in the separation of an indeterminate layer at the ether/water interface. Since this represents a potential loss of material it has been examined. At *p*H 6.0, the ether extracts usually separate well. In a typical experiment with urine at *p*H 7.0 it was found that the indeterminate layer contained 10 μg. propylthiouracil together with some uric acid.

In such instances, where an indeterminate layer forms at the interface, the best method is to combine it with the ether extracts, and then remove it with the water used to wash them.

Treatment of Residue.—The residue left after removal of the ether should be as dry as possible in order to effect its solution in absolute ethanol. The concentration of the latter should not exceed 2 ml. per 100 ml. of water used to make the solution to volume ; 2 ml. of absolute ethanol will dissolve up to 5 mg. of propylthiouracil. Amounts of the latter in excess of this may be dissolved in larger amounts of ethanol, and a proportionate increase made in the water.

Recovery of Propylthiouracil.—With various amounts of propylthiouracil added to urine, the recovery values obtained with the method averaged 85%.

Interfering Substances.—A large number of normal and pathological urines containing no propylthiouracil have been examined by the method and no interfering compounds have been detected. Gibbs (1927a, b) has shown that the chloroimide reagent is a general test for phenols unsubstituted in the para-position, with which it forms blue or violet indophenol pigments. Whether phenols of this type give coloured products, which are removable from the reaction mixture at *p*H 8.0 by means of chloroform, has not as yet been determined, but, as Fearon (1944) has pointed out, p-cresol, the commonest urinary phenol, does not give a colour with the reagent, and other urinary phenols are almost entirely present in fresh urine in an esterified non-reacting form.

The commoner pathological constituents of urine such as proteins, acetone, acetoacetic acid, do not interfere in the reaction.

Excretion of Propylthiouracil.—In patients receiving 100 mg. of propylthiouracil a day, urinary levels of the drug obtained by the method averaged 52 mg. a day.

Summary

A specific colour reaction for antithyroid compounds of the thiouracil type has been described, and a method for the determination of propylthiouracil in urine given.

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A SIMPLIFIED METHOD FOR ESTIMATING CARBON MONOXIDE IN BLOOD WITH THE VOLUMETRIC VAN SLYKE APPARATUS

BY

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The volumetric methods for the estimation of blood gases, for example, those of Van Slyke and Salvesen (1919), O'Brien and Parker (1922), Hawk, Oser, and Summerson (1947), are inferior in accuracy to the manometric, for example, that of Van Slyke and Neill (1924), but the simplicity and lower cost of the volumetric apparatus more than outweigh this disadvantage where the determination of carbon monoxide saturation for clinical purposes is required. The volumetric method, however, usually involves a number of difficulties which are especially troublesome to those who only use it occasionally ; some of these difficulties also apply to the manometric. It is necessary, for example, for the carbon-dioxide-absorbent, and if carbon monoxide is to be measured by absorption the oxygen-absorbent as well, to be gas-free. It is difficult to determine when the vacuum extraction is complete, extraction to constant volume not being practicable owing to the lack of fine graduations above 1.0 ml. The extraction is in any case an equilibrium procedure, and the solution will ultimately be brought into equilibrium with an atmosphere whose pressure and composition vary with variations in the gases extracted ; allowance for this complicates the calculation or renders empirical corrections desirable. Most important of all, as there is no opportunity to clean out the apparatus during an estimation, traces of the original blood remain in the chamber and gas-burette and may cause precipitates with cuprous chloride or the reabsorption of CO when haemoglobin is re-formed by hydrosulphite. Scholander and Roughton (1942) have described a simplified micro-gasometric method for estimating CO. This gives reasonably accurate results, the standard deviation of differences between their published results and determinations by Horvath and Roughton's (1942) manometric method being 0.93, and replicate determinations always being within 2% CO-saturation of each other. As, however, corrections are left to balance themselves, it must be considered as an empirical method and greater reliance placed on more orthodox Van Slyke techniques. Harington and Van Slyke (1924) have modified the chamber of the manometric apparatus, thus allowing it to be washed out during a determination and so enabling cuprous chloride to be introduced without causing precipitates. Van Slyke and Robscheit-Robbins (1927) have also described a method for the determination of CO using this modification. The alteration consists of the addition of an extra bulb, stopcock, and exit tube below

the chamber. Its construction will be beyond the capabilities of many laboratories, and it does not avoid the exposure of solutions to a greatly reduced pressure between the absorption processes.

My purpose is to describe a simple attachment to the volumetric Van Slyke apparatus, which can be made by any technician, and which will permit gases to be ejected from and returned quantitatively to the chamber. It is therefore possible to wash the latter out at any time, and as the extracted gases need never be reduced appreciably below atmospheric pressure there is no need for absorbing solutions to be rendered gas-free. The method is presented as reasonably accurate and rapid, but above all as a reliable and convenient technique, which will give dependable results in the hands of a worker only occasionally having to use it.

Apparatus and Materials

A bulb is blown in a piece of 2-mm. bore manometer tubing having a capacity of about the same as the greatest volume of blood sample it is proposed to use (i.e., 2 ml., or for special purposes 5 ml.). This tubing is bent at the top and attached to the bent capillary of the Van Slyke (Fig. 1). Its lower end dips vertically into a large, thick-walled test-tube containing mercury, and the tube and bulb are themselves filled with mercury before an analysis. It is obviously preferable that the joint should be a fused one, but failing this it may be made with translucent polyvinyl chloride ("P.V.C.") tubing, the glass ends being brought as closely into apposition as possible. P.V.C., unlike rubber, tubing has the advantages of being non-porous and of permitting air-bubbles to be seen through it. It should be stated, however, that in practice no trouble from the retention of air-bubbles at the joint has been encountered, and that all results recorded in this paper were obtained with an apparatus with a P.V.C. joint. If an apparatus is being specially constructed it should be noted that the modification renders the two limbs below the lower stopcock superfluous, and that the tubing connexion can be placed immediately below a single-bore stopcock. The use of P.V.C. tubing for connecting the levelling bulb should render the use of a Shohl air-trap unnecessary.

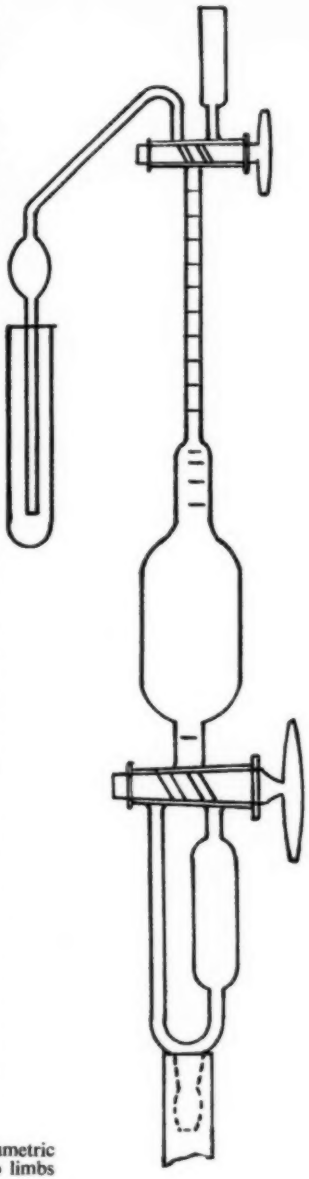

FIG. 1.—Modification applied to standard Van Slyke volumetric apparatus. (In a specially constructed apparatus the two limbs below the lower cock could be eliminated.)

The whole apparatus is rigidly supported on a steel retort stand, the base of which is clamped to the bench. Agitation is performed by maintaining a rotary vibration of the ½-in. rod of the stand, which will swirl the solution over the chamber walls without throwing mercury out of the test-tube. The use of a silicone* grease for lubricating stopcocks is strongly recommended, as it eliminates all difficulties arising from organic greases being attacked by the strong alkalis used. It is also recommended for greasing the stoppers of the reagent bottles containing the alkaline absorbing solutions.

If measurements in terms of volumes per cent are required Ostwald-Van Slyke pipettes are used for measuring the blood. These are tipped, not with the shaped piece of thick rubber tubing usually recommended, but with a thin unshaped piece of tubing (4 mm. diameter, 2 mm. bore) which takes the shape of the tip of the pipette and fits snugly in the mouth of the capillary below the cup. If results are required as percentage saturation, less precision is of course necessary in measuring the sample, as its volume will only enter into the calculation of the correction for dissolved oxygen. Here an 8% error in the volume of blood will only make a difference of 0.001 ml. to the correction. Ordinary pipettes can therefore be used so long as care is taken that no air follows the blood into the chamber.

Reagents.—The following are required.

(1) Acid Ferricyanide.—This is described by Hawk *et al.* (1947).

Saponin	3.0 g.
Potassium ferricyanide	8.0 g.
Lactic acid	4.0 ml.
Capryl alcohol	3.0 ml.
Distilled water to	1,000 ml.

The reagent should be shaken before use. It is stable for several weeks.

(2) Potassium Hydroxide (approximately N).

(3) Potassium Hydroxide (approximately 4N).

(4) Hydrosulphite Reagent.—This is made up as follows:

Sodium hydrosulphite ($Na_2S_2O_4$)	5.0 g.
Sodium anthraquinone 2-sulphonate	0.5 g.
Potassium hydroxide approximately N	25 ml.

This should be made, with the minimum exposure of the solution to the air, in the bottle in which it is to be stored. It is stable in a well-stoppered bottle for about a week. When the colour changes from red to yellow it should be discarded.

(5) Ammoniacal Cuprous Chloride.—This contains the following:

Cuprous chloride	40 g.
Ammonium chloride	50 g.
Distilled water to	150 ml.
Ammonia (S.G. 0.9)	50 ml.

The reagent should be stored in a well-stoppered bottle over copper wire, when it will be stable for several weeks.

(6) Ammonia, approximately 3N.

(7) Lactic acid, approximately N.

* For example, Dow-Corning stopcock grease, supplied in England through Messrs. W. Edwards and Co. (London), Ltd.

Procedure

The apparatus is first rinsed out with the ferricyanide reagent. Then 6 ml. of reagent is introduced into the chamber and extracted repeatedly until gas-free, the gases being ejected via the cup after each extraction. The blood sample (usually 2 ml.) is then introduced under 1 ml. of mercury in the cup, and is extracted repeatedly until no more gas is evolved, extracted gases being passed into the side-tube after each extraction. After ejection of the blood via the cup the gas burette and chamber are washed out with N KOH; 0.58 ml. 4N KOH are introduced into the burette, and the gases returned. The fluid level is lowered repeatedly to about the 1.5-ml. mark until no further absorption of carbon dioxide takes place; the volume of oxygen plus carbon monoxide plus nitrogen is then read over the KOH column, allowing for the density of the latter in levelling as mentioned below. Readings are made with a lens, with a good light behind the burette, interpolating to 0.001 ml. The gases are then returned to the side-tube and the KOH ejected via the cup. Hydrosulphite reagent, 0.58 ml., is run into the burette, gases returned for absorption of oxygen, and the volume of carbon monoxide plus nitrogen read over the reagent column as before. The remaining gases are again returned to the side-tube, and the reagent ejected via the cup. The burette and entry to the side-tube are washed twice with water, and 0.58 ml. of cuprous chloride introduced into the former. Gases are returned for absorption of CO, and the volume of residual nitrogen read over the reagent. Finally the apparatus is cleaned and prepared for the next determination by washing out both chamber and side-tube with dilute ammonia, followed by N lactic acid. All absorptions are rapid, and take only a minute or two.

It should be noted that although normally control of the rate of flow of either a gas or liquid is exercised by the bottom (mercury) cock, and the top one is used only to determine the direction, rather different considerations obtain when it is desired to expel the last traces of gas from the side-tube into the burette. Here the flow of mercury should be interrupted suddenly by means of the top cock, before the gas has time to rise through it.

The absorbent solutions used are of approximately the same density, and their volume is adjusted so that provided the mercury meniscus is not below 1.0 ml. it is only necessary to hold the levelling bulb at a graduation 0.05 ml. higher to allow for the weight of the column of reagent. If the mercury should be in the wider part of the burette it is necessary either to estimate 1/11.5 of the aqueous column, or to eject the latter before reading. In the determination of CO-saturation it is unnecessary to correct for temperature or barometric pressure so long as these have remained constant throughout the analysis, the CO-saturation being calculated as:

$$\text{Volume of CO} \times 100 / (\text{Volume of CO} + \text{Volume of combined } O_2)$$

If results are required in volumes per cent the usual corrections must, of course, be applied.

Correction for Dissolved Gases

O'Brien and Parker have shown that the correction for carbon monoxide in physical solution is negligible. The blood will, however, be saturated with oxygen. Allowing an absorption coefficient of 0.031 for oxygen at 20° C., a factor of 0.92 for the fact that whole blood is used instead of water, 20.9% for the oxygen content of the air, 760 mm. for the barometric pressure, and 20° C. for the temperature, the volume of oxygen in solution in 2 ml. of blood will be 0.013 ml. (measured moist); this value should therefore be subtracted from the measured volume of oxygen. Variation in this correction with temperature is slight (Table I), and variation with barometric pressure is negligible.

TABLE I

CORRECTION FOR DISSOLVED OXYGEN*

Temperature (° C.)	15	20	25	30
Dissolved O_2 (ml.)	0·014	0·013	0·012	0·012

*Blood samples were of 2 ml.

Precision and Accuracy

A specimen of oxalated human venous blood was divided into two parts. One of these was shaken with carbon monoxide, and after bubbles of gas had been dispersed was mixed with the other part and allowed to stand for 24 hours in a closed container to ensure that cells, plasma, and gas phase were in equilibrium. No attempt was made to obtain solutions of known saturation owing to the difficulty of fully saturating whole blood and the possibility of introducing excess of CO in physical solution. Samples, each of 2 ml., were analysed by the method described, with the results given in Table II. It will be observed that the standard deviation is 0.6% CO Hb.

TABLE II

REPRODUCIBILITY (REPLICATE DETERMINATIONS)

Experiment	CO Combined O_2 (ml.)	CO (ml.)	CO Saturation (%)
1	0·451	0·075	16·6
2	0·426	0·075	17·6
3	0·446	0·080	17·9
4	0·438	0·080	17·7
5	0·438	0·075	17·1
6	0·431	0·079	18·3
		Mean	17·5
		Standard deviation	0·6

Ten other partially saturated blood samples were prepared in a similar manner and analysed by the present method and an abridged spectrophotometric method (Nicholas, in press); the range of saturation covered was 14–52%. The mean difference between results was 0.19% CO Hb, with a standard deviation about this mean of 0.69%. Results will be given in full in the reference cited. With two methods so entirely different in principle it is highly improbable that any extraneous factor can have affected both equally, and therefore each can be used as a measure of the reliability of the other. It can therefore be assumed that the standard deviation of either method taken alone does not exceed 0.69% CO Hb, and it is more probable that the errors will be divided between the two methods, giving, if the division is equal, a standard deviation for each of $0.69/\sqrt{2}=0.49\%$ CO Hb.

From the foregoing the possible error of the method can be considered as of the order of $\pm 1.0\%$ saturation ($2 \times$ S.D.). This compares favourably with Van

Slyke and Salvesen's figures, from which a mean deviation from expectation of 0.77% saturation and a standard deviation about this mean of 1.33% saturation can be calculated. It is possible that some of their error may be accounted for by unavoidable inaccuracy in the preparation of the blood mixtures, but in the two cases where they did duplicate estimations they obtained differences of 0.9% and 2.2% saturation.

Summary

A modification of the Van Slyke volumetric apparatus is described which provides storage space for gases, thus enabling the chamber and gas burette to be washed out in the course of an estimation, and dispensing with the exposure of gas-absorbing reagents to a vacuum ; the latter do not, therefore, require to be gas-free. The modification of the apparatus can be carried out in any laboratory.

A method is described for the estimation of carbon monoxide using this apparatus, which can be considered as accurate to ±1.0% CO saturation.

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THE REACTION OF HAEMOPHILIC PLASMA TO THROMBOPLASTIN

BY

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Two well-known observations on the clotting defect of haemophilic blood would seem to be of basic importance. The first is that haemophilic blood responds quite normally to a strong suspension of tissue thromboplastin ; the second, that it will clot in practically the normal time on the addition of a small proportion of normal plasma. The normal response to thromboplastin naturally suggests that the part of the clotting mechanism which is set in motion by thromboplastin is in normal working order. Certainly no defect has been demonstrated in haemophilic prothrombin, fibrinogen, or calcium, and the various factors recently described as being concerned in prothrombin conversion, such as Factor V (Owren, 1947) and " serum prothrombin conversion accelerator " (Alexander and de Vries, 1949), are stated by these respective authors to be normal in haemophilia.

The essential defect in haemophilic blood, therefore, seems to be its inability to clot normally in the absence of added thromboplastin, a defect which is largely corrected by the addition of a small amount of normal plasma. Since normal blood clots rapidly in glass, even when obtained with a minimum of contamination with tissue fluid, it must be supposed that there is some intrinsic mechanism for the production of thromboplastic activity. This matter has been discussed by many workers, and two current views are put forward by Brinkhous (1947) and Quick (1947) respectively. These authors agree in supposing that intrinsic thromboplastic activity develops on contact with a foreign surface, and depends on interaction between the platelets and a plasma factor. In Quick's view it is the plasma factor, " thromboplastinogen," that is the inactive precursor of thromboplastin and which is activated by a platelet factor. Brinkhous considers that the platelets themselves are the source of thromboplastin, and that they are activated by being broken up by the plasma factor, which is a " thrombocytolysin." It is agreed by both that the platelets are normal in haemophilia, and that it is the plasma factor which is deficient in this condition. Normal plasma contains enough of this " antihaemophilic factor " to be able to correct the deficiency.

Ignoring the slight divergence of views as to the mode of action of the anti-haemophilic factor, it seems to be accepted that its deficiency in haemophilia results in a deficient production of intrinsic thromboplastin, such thromboplastin presumably having a similar action to that of the more familiar tissue thromboplastin.

The normal response of haemophilic blood to added tissue thromboplastin is clearly important to the validity of this hypothesis. There is little doubt that with

strong suspensions of brain extract thrombin is formed in the same amount and at the same speed in haemophilic as in normal plasma or from haemophilic prothrombin and Factor V as compared with normal factors (Biggs, 1951b). Quick (1947) has claimed that haemophilic plasma reacts normally to even minute amounts of thromboplastin, but other workers would not agree with this statement. Addis, in 1911, showed that haemophilic plasma clotted more slowly than normal with added testis or kidney thromboplastin, and more recent observations suggest that its reaction to weak brain suspensions is less rapid than normal (Pavlovsky, Mittelman, and Castellanos, 1949; Dreskin and Rosenthal, 1950; Frommeyer, Epstein, and Taylor, 1950).

In view of this uncertainty it was considered that a study of the reactions of normal and haemophilic plasma and plasma fractions with different concentrations of thromboplastic agents might provide further information on the mode of action of the "antihaemophilic factor."

Technique

Reagents.—The following reagents were used:

Brain Thromboplastin.—Fresh human brain is collected in the post-mortem room, and all superficial vessels and meninges are removed. The substance is macerated with three or four times its volume of acetone in a mortar. The acetone is replaced four times, and the granular powder is dried by means of a suction filter. The dried brain can be stored for periods up to three months at room temperature with no special precautions.

For use 0.5 g. of the granular powder is suspended in 5 ml. of saline and incubated at 37° C. for 15 minutes. The suspension is mixed once or twice during this time, and the coarse particles are allowed to settle by gravity. The supernatant fluid is removed and diluted 1/5 with 0.85% saline.

Calcium Chloride.—An M/40 solution of anhydrous calcium chloride is used.

Fibrinogen (Jaques, 1943).—First a 3 M phosphate buffer at *p*H 6.6 is made by dissolving 817 g. of anhydrous KH_2PO_4 in 1,000 ml. of distilled water. To this is added 750 ml. of 4N KOH. After warming, the volume is made up to 2,000 ml. and the solution is filtered.

Secondly, a preparation of $Al(OH)_3$ suspension (Bertho and Grassmann, 1938) is made up as follows: 100 ml. of 10% ammonia solution is poured into 600 ml. of water at 63° C. containing 22 g. of ammonium sulphate, and the temperature is rapidly brought to 58° C. The mixture is stirred vigorously and poured, in one lot, into a solution of 76.7 g. of ammonium alum at a temperature of 58° C. The temperature rises to 61° C. Stirring is continued for 10 minutes, during which time the temperature is maintained above 58° C. The precipitate is separated by centrifuging. The precipitate is washed five times with 1,500 ml. of distilled water, separating the precipitate by centrifuging on each occasion. To the first washing water is added 0.44 ml. of 10% ammonia, and to the second 0.88 ml. of 10% ammonia. The whole procedure does not take much more than two hours. The precipitate is suspended in the least amount of distilled water that is required to make a solution that can be pipetted.

Thirdly, the fibrinogen is prepared. To 100 ml. of citrated plasma obtained from "bank" blood is added 2 ml. of $Al(OH)_3$ suspension to remove the prothrombin. The $Al(OH)_3$ is removed by centrifuging and discarded. To 100 ml. of this prothrombin-free plasma is added 100 ml. of 2 M phosphate buffer at *p*H 6.6. After centrifuging, the precipitate is washed with 100 ml. of molar phosphate buffer and dissolved in 75 ml. of M/4 phosphate buffer. The precipitation is repeated twice and the final precipitate is

dissolved in 50 ml. of 0.85% saline containing 0.0075 M sodium citrate. The solution is dialysed for 12 hours against citrate saline. The concentration of fibrinogen is usually about 400 mg. %.

Collection of Blood.—Blood was collected from normal and haemophilic subjects by venepuncture, and coagulation prevented by mixing 9 parts of blood with 1 part of 3.8% sodium citrate. The blood was centrifuged at 2,000 r.p.m. for 15 minutes. The plasma was separated for use.

Preparation of Fractions by Acid Precipitation.—The following methods were used :

"*Globulin Fraction.*"—Plasma was dialysed overnight against 0.38% sodium citrate. The *p*H was then adjusted to 5, using 10% acetic acid ; the resulting precipitate was removed by centrifuging and dissolved in 0.85% saline, containing 1 part of 3.8 sodium citrate to 9 parts of saline. The volume of the final solution was made about half that of the original plasma. If samples from normal and haemophilic blood were compared, care was taken that the concentrations of globulin were as nearly comparable as possible. Before use the *p*H was adjusted to 7 with 2% sodium carbonate.

"*Albumin Fraction.*"—The supernatant remaining after acid preparation of the globulin fraction was neutralized with 10% sodium carbonate and constituted the albumin fraction. This fraction contains a large amount of protein precipitate by 50% saturation with ammonium sulphate and thus contains protein other than albumin.

Preparation of Fractions by Ammonium Sulphate Precipitation.—This procedure entailed preparing three fractions.

Fibrinogen Fraction (*Fraction I*).—Plasma, 10 ml., was largely freed from platelets by centrifuging for half an hour at 3,000 r.p.m. To the clear plasma were added 2 g. of solid ammonium sulphate. The mixture was well shaken to ensure the complete solution of the ammonium sulphate and the precipitate removed by centrifuging. The precipitate was dissolved in 5 ml. of 0.85% saline and dialysed overnight against saline containing 1/10 of its volume of 3.8% sodium citrate.

Globulin Fraction (*Fraction II*).—A further 1 g. of solid ammonium sulphate was added to the supernatant from the preparation of Fraction I. The precipitate was collected by centrifuging and dissolved in 5 ml. of 0.85% saline and dialysed against citrate saline.

Albumin Fraction (*Fraction III*).—The supernatant from the preparation of Fraction II was dialysed against citrate saline.

Technical Methods

One-stage Prothrombin Time.—Plasma, 0.1 ml., is mixed with 0.1 ml. of thromboplastin, and the mixture warmed to 37° C.; 0.1 ml. of M/40 calcium chloride is then added and the coagulation time recorded.

Two-stage Prothrombin Test.—A modification of the method of Herbert (1940) and Owren (1947) was used. Plasma, 0.3 ml., and 0.3 ml. of saline were mixed with 0.3 ml. of brain emulsion, and the mixture was warmed to 37° C. Then 0.3 ml. of M/40 calcium chloride was added, and at various intervals from one to 30 minutes 0.1 ml. samples were removed and added to 0.4 ml. amounts of fibrinogen in small tubes previously prepared and placed in the water bath. The coagulation times of the fibrinogen were recorded. The concentration of fibrinogen was adjusted to approximately 100 mg. %.

If thrombin formation was followed in the globulin fraction instead of whole plasma, 0.3 ml. of globulin fraction was mixed with 0.3 ml. saline or 0.3 ml. of the "albumin fraction" and 0.3 ml. of brain emulsion. Then 0.3 ml. of M/40 $CaCl_2$ was added, and thrombin generation was followed as before.

The clotting times obtained by the two-stage method record the concentrations of thrombin in the incubation mixture. The concentrations can be expressed as relative units of thrombin using a calibration curve prepared by adding decreasing concentrations of a solution of thrombin containing 1,000 units per ml. to 0.4 ml. samples of fibrinogen and recording the clotting times. For the graphical representation of results the clotting times have been converted to arbitrary thrombin units by this method.

Experimental Results

Experiments on Whole Plasma.—A few experiments were made using the one-stage method. Using normal and haemophilic plasma samples, the one-stage method was carried out with dilutions of brain emulsion varying from 1/10 to 1/50,000. The clotting time obtained with each sample of haemophilic plasma was then plotted against the clotting time of the corresponding normal plasma obtained with the same thromboplastin preparation. From Fig. 1 it will be seen that when the thromboplastin preparation was diluted to give with normal plasma a clotting time of more than 40 seconds, usually the haemophilic plasma had a longer clotting time than the normal. Thus, with quite strong suspensions of thromboplastin, the reaction of haemophilic plasma was abnormal.

This abnormality might be attributed to an initial deficiency of thromboplastin in the haemophilic plasma due to its supposed inability to generate intrinsic thromboplastin activity. If this is the true explanation, then an appropriate addition of thromboplastin to the haemophilic plasma should correct the defect. Enough thromboplastin was added to haemophilic plasma to make its clotting time the same as that of the normal plasma to which no thromboplastin had been added. The amount required was a 1/10,000 concentration of brain emulsion. Further additions of thromboplastin were then made to both normal and haemophilic plasma. From Fig. 2 it will be seen that the further additions of thromboplastin had less effect on haemophilic than on normal plasma. The defect in haemophilic plasma therefore cannot be attributed to a simple deficiency in thromboplastin.

The long clotting time of haemophilic plasma might be due to its neutralization of thromboplastin. A number of samples of normal and haemophilic plasma were incubated with brain thromboplastin for one hour, and at intervals samples were recalcified. The clotting time of both normal and haemophilic samples lengthened slightly, but there was no striking difference in their behaviour on incubation, though the clotting times of the haemophilic plasma samples were consistently longer than those of the normal (Fig. 3). In a further experiment one normal and two samples of haemophilic plasma were incubated at 37° C. for one hour with two strengths of brain thromboplastin. At the end of this time the samples were used undiluted and diluted 1/10 as a source of thromboplastin for the one-stage method. The samples were tested on the same normal and the same two samples of haemophilic blood in all combinations. The results of this experiment are set out in Tables I and II. Incubation of brain thromboplastin with the normal plasma in this experiment led to rather more inactivation of thromboplastin than did incubation with either of the haemophilic plasmas. From these experiments it seems probable that progressive inactivation of brain thromboplastin by plasma is not an essential feature of haemophilic plasma.

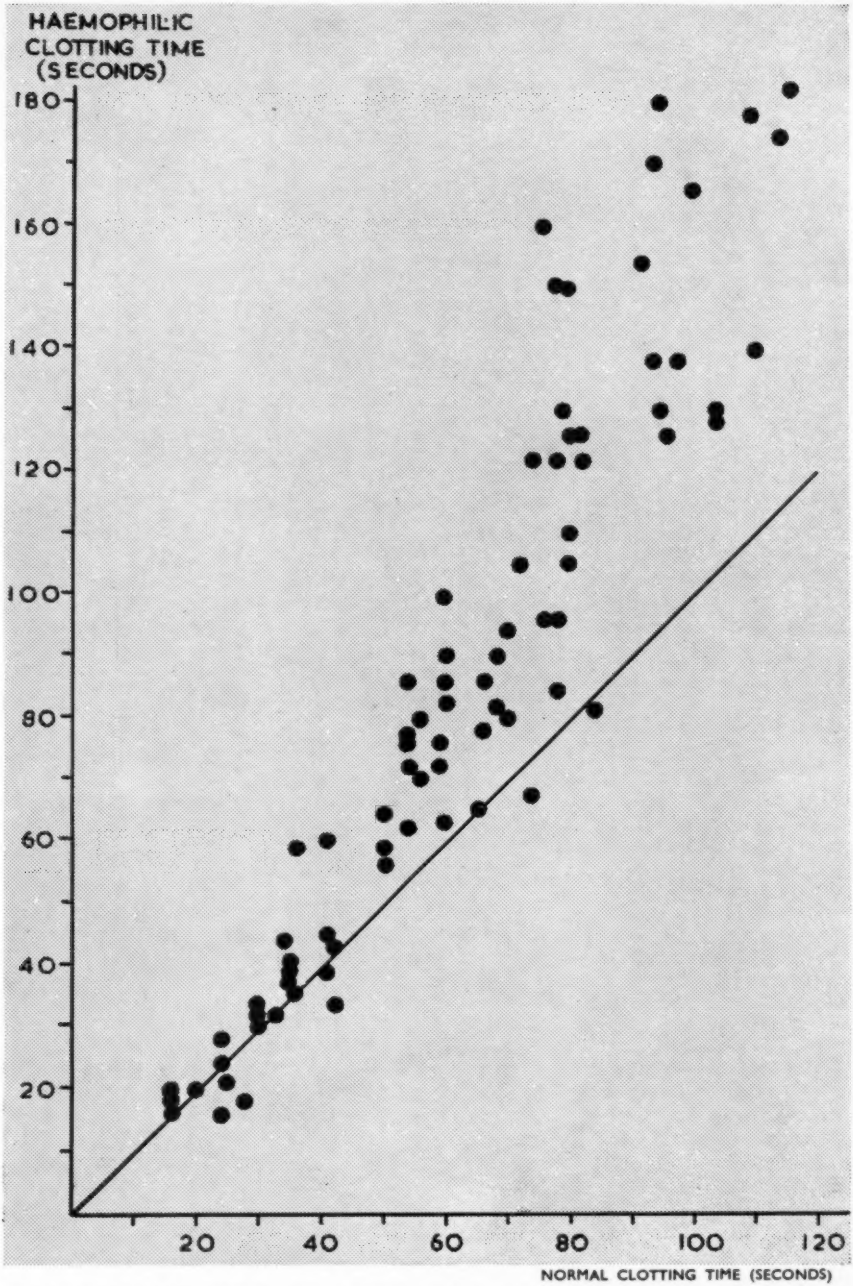

FIG. 1.—Normal and haemophilic plasma samples were tested by the one-stage method using various dilutions of brain emulsion as thromboplastin. In the diagram the clotting times obtained with normal plasma samples are plotted against the clotting times obtained with the same thromboplastin preparations with haemophilic plasma samples. With concentrated preparations of thromboplastin which give clotting times with the normal plasma of less than 40 seconds, the clotting times of the haemophilic samples are often the same as normal; with weaker suspensions of brain emulsion the clotting times of the haemophilic samples are longer than those of the normal plasma samples.

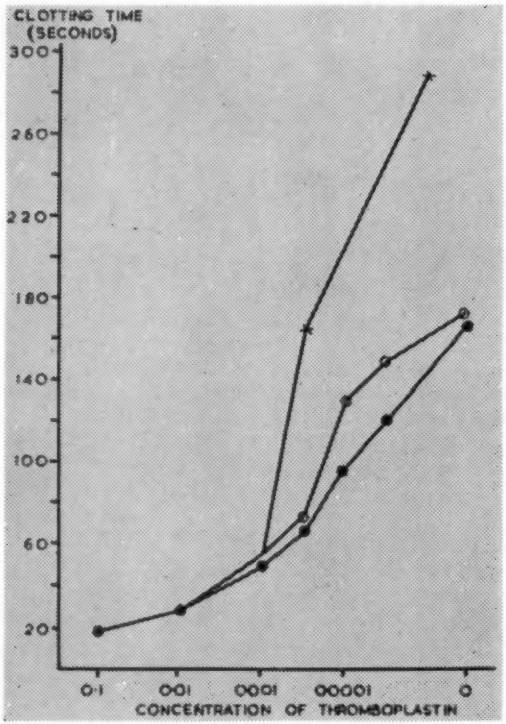

FIG. 2.—The results of the one-stage method carried out with various dilutions of brain emulsion varying from 1/10 to 1/10,000 with (1) normal plasma (●), (2) haemophilic plasma (×), (3) haemophilic plasma containing a 1/10,000 concentration of brain emulsion (○).

FIG. 3.—Samples, each 0.1 ml., of normal and haemophilic plasma were incubated at 37° C. for periods up to one hour with various concentrations of brain emulsion. The clotting times of these mixtures on recalcification were tested. In any particular experiment the normal and haemophilic plasma samples were incubated with the same preparation of brain emulsion. The results represent the average for seven independent experiments. The upper curve shows the clotting times of the haemophilic plasma samples and the lower curve the clotting times of the normal plasma samples.

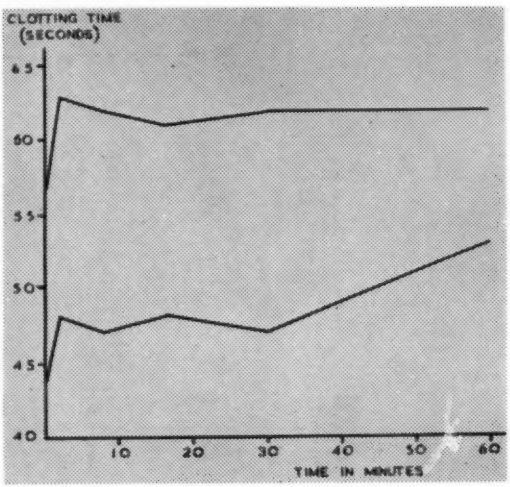

TABLE I

CLOTTING TIMES DERIVED FROM ONE-STAGE TEST (Q.V.) OF NORMAL AND HAEMOPHILIC PLASMAS WITH THROMBOPLASTIN USING DIFFERENT PLASMAS AS SUBSTRATES

Dilution of Brain Emulsion Incubated with Plasma	Type of Plasma	Clotting Times (secs.)		
		Normal	Haemophilic I	Haemophilic II
1/5	Normal	22	17	26
	Haemophilic I	17	16	20
	Haemophilic II	21	17	28
1/50	Normal	51	35	45
	Haemophilic I	31	30	33
	Haemophilic II	35	30	41

Three plasma samples, one normal and two from haemophilic patients, were incubated at 37° C. for one hour with an equal volume of brain emulsion diluted 1/5 and 1/50 with 0·85% saline. At the end of the hour the mixtures were used as a source of thromboplastin.

TABLE II

CLOTTING TIMES DERIVED FROM ONE-STAGE TEST (Q.V.) OF NORMAL AND HAEMOPHILIC PLASMAS USING THE SAME PLASMAS AS SUBSTRATE

Dilution of Brain Emulsion Incubated with Plasma	Type of Plasma	Clotting Times (secs.)		
		Normal	Haemophilic I	Haemophilic II
1/5	Normal	31	24	40
	Haemophilic I	32	23	30
	Haemophilic II	38	28	37
1/50	Normal	75	63	79
	Haemophilic I	58	54	66
	Haemophilic II	58	54	68

Three plasma samples, one normal and two haemophilic, were incubated at 37° C. for one hour with an equal volume of brain emulsion diluted 1/5 and 1/50 with 0·85% saline. At the end of the hour these samples were diluted 1/10 with 0·85% saline, giving final dilutions of brain emulsion of 1/50 and 1/500 and used as a source of thromboplastin.

It is possible that haemophilic plasma contains an inhibitor of thromboplastic action which requires the presence of calcium or which is inhibited by sodium citrate. This possibility was not investigated, because it is difficult to study the destruction of thromboplastin in a system which is forming thrombin and a serum accelerator of thrombin formation.

In the majority of experiments thrombin formation was followed by the two-stage method in a system containing the albumin fraction which has a potent anti-thrombic action. Thrombin was therefore destroyed progressively as it was formed, and it is important to appreciate the factors which influence the concentration of thrombin in a system of this sort. The amount of thrombin available depends on the relative speeds of its formation and destruction.

The effect of varying the speed of formation may be shown in an experiment in which the amount of thromboplastin added to plasma in the two-stage method is varied. The stronger suspensions of thromboplastin lead to rapid thrombin generation, and a high level of thrombin is detected. With slow speeds of generation much less thrombin can be demonstrated (Fig. 4), although the same total amount is probably produced.

FIG. 4.—The curves represent the progress of thrombin formation and disappearance followed by the two-stage method in the same sample of normal plasma using brain preparations of varying strength. The curve o represents the reaction with brain emulsion diluted 1/10; the curve × the reaction with brain emulsion diluted 1/50; the curve ● the reaction with brain emulsion diluted 1/100.

FIG. 5.—The curves represent the progress of thrombin formation followed by the two-stage method in normal plasma diluted 1/10 with 0.85% saline (upper curve) and in the same plasma diluted with 0.85% saline to which various concentrations of plasma adsorbed with $Al(C_6H_5)_3$ had been added.

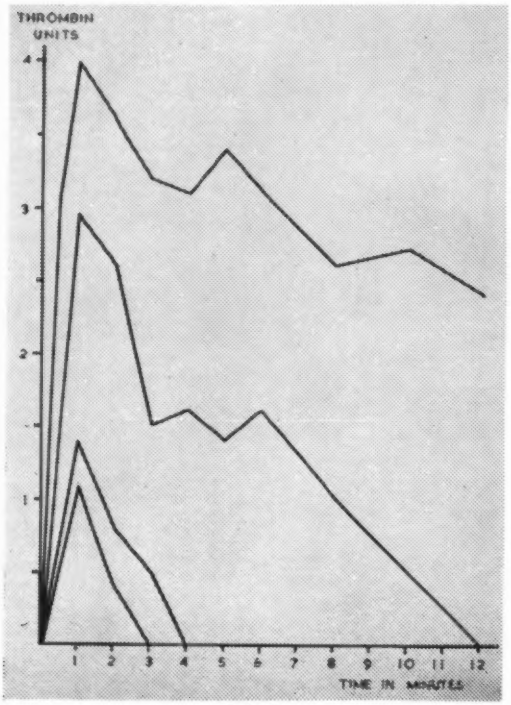

FIG. 5

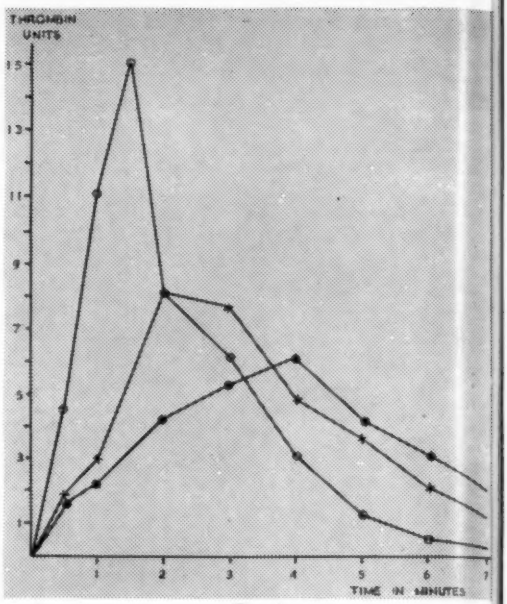

FIG. 4

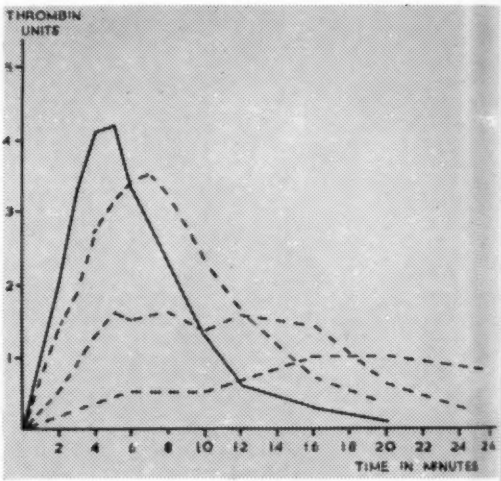

FIG. 6

FIG. 6.—The curves represent the progress of thrombin formation in the presence of dilute brain thromboplastin followed by the two-stage method in normal plasma (continuous line) and three samples of haemophilic plasma from different patients.

The speed of production may be kept constant and the activity of the antithrombin may be increased by adding to a saline dilution of plasma plasma adsorbed with $Al(OH)_3$ to remove prothrombin. When plasma prepared in this way is tested by the two-stage method the curves in Fig. 5 result. Similar curves result from the addition of heparin to plasma. The amount of thrombin detected is controlled both by the speed of thrombin formation and the speed of its destruction.

In a number of samples of haemophilic plasma thrombin formation was studied by the two-stage method, using a 1/100 or 1/1,000 dilution of brain thromboplastin. In Fig. 6 the results in three individual haemophilic plasmas are compared with the average for four normal plasmas. From the previous discussion it is probable that the difference between the haemophilic and normal plasmas lies mainly in a difference in the speed of thrombin formation.

The addition of normal plasma to haemophilic whole plasma greatly improves the reaction of haemophilic plasma to weak suspensions of brain thromboplastin. In Fig. 7 are shown the effects on thrombin formation of adding 1/10 and 1/2 proportion of normal plasma to haemophilic plasma. These results are the mean values for eight experiments on the blood of different haemophilic and normal people. It will be seen that 1/10 volume of normal plasma considerably increases the speed of thrombin formation, but that even 50% of normal plasma does not restore thrombin formation to normal. The effect of 1/10 dilution of normal plasma is worth emphasizing. In previous experiments it was found that a concentration of 1/10,000 brain emulsion was required to bring the one-stage clotting time of haemophilic plasma to the same as that of normal plasma. Therefore, if the defect in haemophilic plasma is due to a deficiency in thromboplastin, normal plasma must contain an excess of thromboplastin in comparison with haemophilic plasma, and this excess is approximately equal to a brain concentration of 1/10,000. The addition of 1/10 proportion of normal to haemophilic plasma would therefore be equivalent to the addition of 1/100,000 dilution of brain thromboplastin. This concentration of brain thromboplastin is practically inactive with haemophilic plasma. This experiment therefore confirms the view that the defect in haemophilia cannot be a simple lack of thromboplastin.

Experiments on Plasma Fractions.—It has been shown previously (Biggs, 1951b) that prothrombin and Factor V isolated from haemophilic plasma have a normal reaction to platelet thromboplastin. It is therefore probable that the abnormal reaction of whole haemophilic plasma to thromboplastin is a feature of whole plasma, which is lost on fractionation. An attempt to identify the cause of the abnormality was made by a more simple fractionation of the plasma. Whole normal plasma and whole haemophilic plasma were dialysed against distilled water, and the globulin fraction precipitated by bringing the *p*H to 5. This fraction was dissolved in saline and neutralized. The supernatant plasma was neutralized and called the albumin fraction. In fact, this fraction was a crude mixture of albumin and globulin. These fractions were tested in various mixtures by the two-stage method, using a weak suspension of brain thromboplastin. The experiment was carried out on five haemophilic and five normal subjects.

The formation of thrombin in the globulin fraction alone given as an average for the five experiments shows no difference between the normal and haemophilic fractions (Fig. 8). This results suggests that the factor causing the abnormal reaction

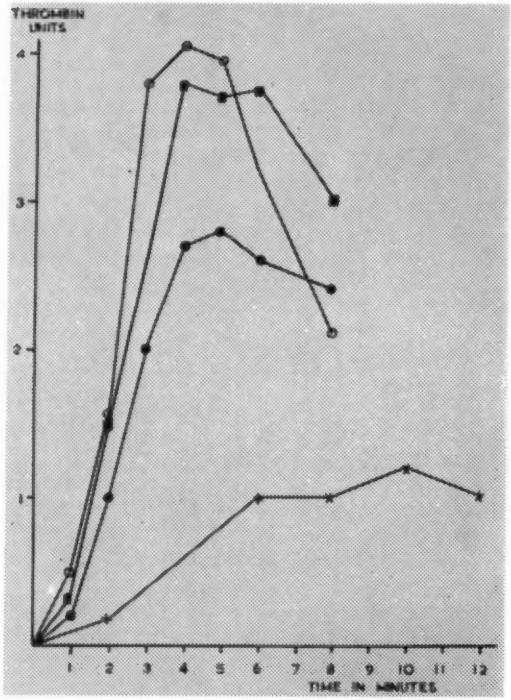

FIG. 7.—The curves represent the progress of thrombin formation in the presence of dilute brain thromboplastin followed by the two-stage method in (1) normal plasma (○), (2) haemophilic plasma (×), (3) haemophilic plasma to which 1/10 of its volume of normal plasma had been added (●), (4) a mixture of haemophilic and normal plasma in equal parts (■). The results are the mean values for eight independent experiments on different samples of normal and haemophilic plasma.

of haemophilic plasma to weak thromboplastin lies in the albumin fraction or in the reaction between factors in the albumin and globulin fractions.

Thrombin formation was then studied in mixtures of the albumin and globulin fractions of normal and haemophilic plasma in all combinations. The speed of thrombin formation was increased by the addition of the normal albumin fraction to the normal globulin fraction and to the haemophilic globulin fraction (Figs. 9 and 10). The addition of haemophilic albumin to the normal globulin also caused an increased speed of thrombin formation (Fig. 9). The addition of haemophilic albumin to haemophilic globulin gave no increase in the speed of thrombin formation. The results illustrated in Figs. 9 and 10 represent the average for five independent experiments on different haemophilic and normal plasmas. The proportions of the different fractions used were constant in all the experiments.

The interpretation of these results is not simple. The method of fractionation failed to separate the better recognized plasma constituents at all specifically. The "globulin fraction" from normal plasma probably contained prothrombin, Factor V, fibrinogen, and some antihaemophilic factor. In two experiments the normal albumin fraction contained the antihaemophilic factor in addition to antithrombin.

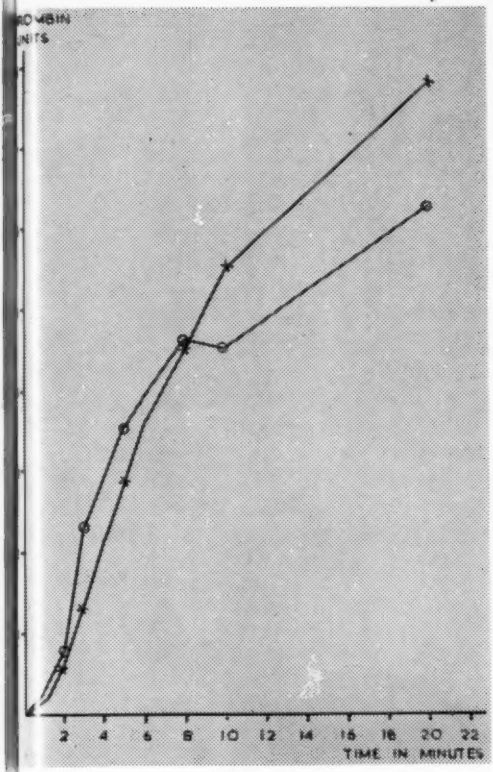

FIG. 8

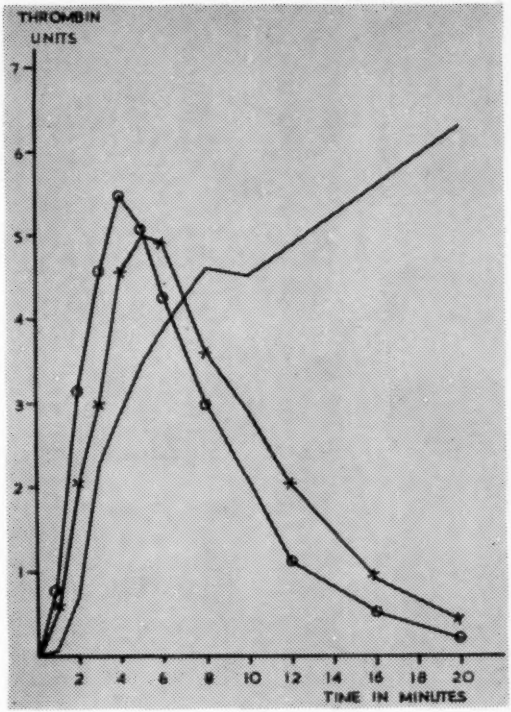

FIG. 9

FIG. 8.—Thrombin formation in the presence of dilute brain thromboplastin in (1) the globulin fraction from normal plasma (O), and (2) the corresponding fraction from haemophilic plasma (×). The curves in Figs. 8, 9, and 10 are the average values for five independent experiments.

FIG. 9.—Thrombin formation and disappearance in (1) the globulin fraction from normal plasma mixed with the albumin fraction from normal plasma (O), and (2) the globulin fraction from normal plasma mixed with the albumin fraction from haemophilic plasma (×). Thrombin formation in the normal globulin fraction alone is given for comparison.

FIG. 10.—Thrombin formation and disappearance in (1) the globulin fraction prepared from haemophilic plasma mixed with the albumin fraction from normal plasma (O), and (2) the globulin fraction from haemophilic mixed with the albumin fraction from haemophilic plasma (×). Thrombin formation in the haemophilic globulin fraction alone is shown for comparison.

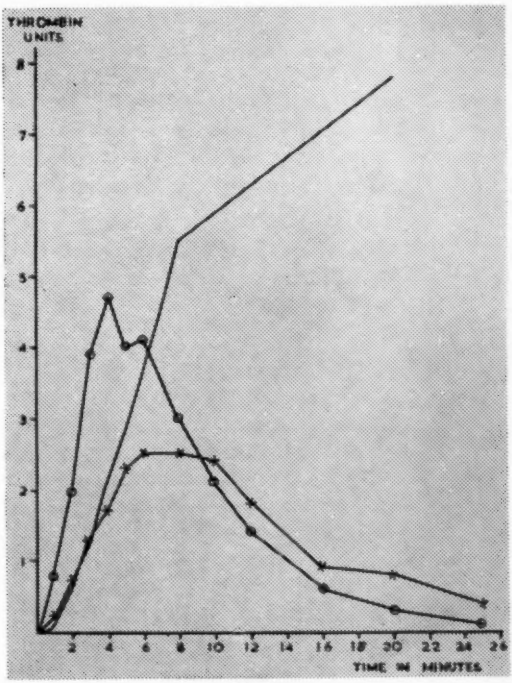

FIG. 10

The fact that the so-called globulin fractions from normal and haemophilic plasma behaved in the same manner, and the fact that the speed of thrombin was accelerated if both the antihaemophilic factor and the albumin fraction were present, suggest that the full effectiveness of the antihaemophilic factor depends in some way on its interaction with some substance in the albumin fraction.

An attempt was made to obtain a more specific separation of important components by ammonium sulphate fractionation. In this way fractions precipitated at 33% and 50% saturation with ammonium sulphate and a supernatant from the 50% saturation were isolated. The 33% fraction (Fraction I, or fibrinogen fraction) from normal plasma contained the fibrinogen and antihaemophilic factor, Fraction I from the haemophilic plasma contained no antihaemophilic factor, the 50% fraction (Fraction II, or globulin fraction) contained prothrombin and Factor V, and the supernatant from the 50% fraction (Fraction III, or albumin fraction) contained antithrombin. The only fraction which showed any ability to shorten the clotting time in haemophilic plasma was the 33% fraction from normal plasma. These fractions were tested for their ability to form thrombin, and in general the results confirm the findings obtained with the acid fractionation. Some of the experiments are illustrated in Figs. 11 and 12.

When tested alone Fractions I and II from normal and haemophilic plasma behaved similarly. In Fig. 11 the three normal fractions were tested together and gave a rapid speed of thrombin generation. When the normal Fraction I was replaced by that from haemophilic blood there was a considerable delay in thrombin formation. When in addition the haemophilic albumin fraction replaced the normal albumin fraction a further reduction in the speed of thrombin formation occurred.

In Fig. 12 similar experiments with the haemophilic fractions are shown. When all three fractions from haemophilic plasma were used thrombin formation was poor. When the haemophilic Fraction I was replaced by that from normal plasma the speed of thrombin formation was accelerated. The further replacement of the haemophilic albumin fraction by the corresponding normal fraction led to a second increase in the speed of thrombin formation.

The fact that the globulin fractions from haemophilic and normal plasma behaved similarly, and that the normal albumin fraction accelerated the speed of thrombin formation from the normal globulin fraction, suggests that the full effectiveness of the antihaemophilic factor depends on its co-operation with a factor in the albumin fraction.

The inhibitory effect of the haemophilic albumin fraction in this experiment may have been due to the presence of an inhibitory substance as a result of immunization following repeated transfusions (Frommeyer *et al.*, 1950), or the haemophilic albumin may have lacked some substance present in normal plasma. The inhibitory effect of the haemophilic albumin fraction is not a consistent finding, because it was observed in one only of the experiments carried out on fractions prepared by acid precipitation.

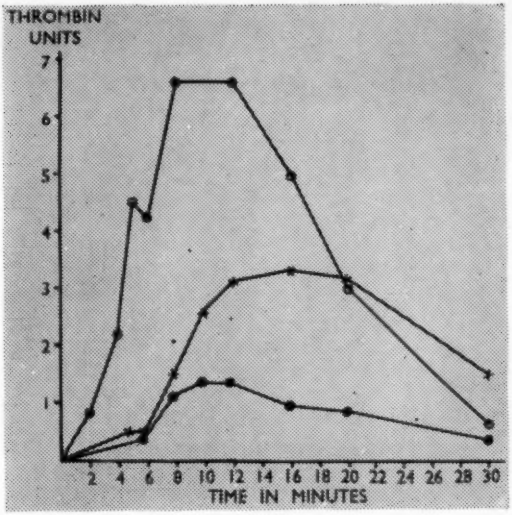

FIG. 11.—The curves represent the progress of thrombin formation followed by the two-stage technique using dilute brain thromboplastin in mixtures of plasma fractions prepared by ammonium sulphate precipitation from haemophilic and normal plasma. In the curve marked ○ the normal fibrinogen, globulin, and albumin fractions were used (Fractions I, II, and III). In the curve marked × the haemophilic fibrinogen fraction was mixed with normal globulin and albumin fractions. In the curve marked ● the haemophilic fibrinogen fraction was mixed with normal globulin and haemophilic albumin.

FIG. 11

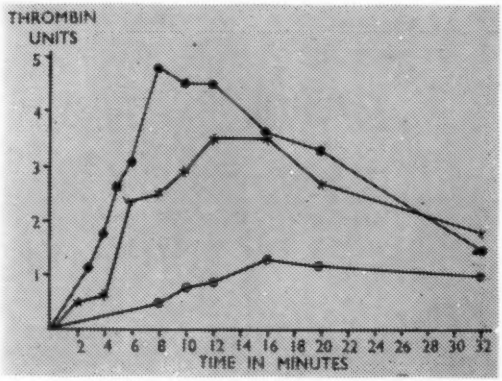

FIG. 12

FIG. 12.—The curves represent the progress of thrombin formation followed by the two-stage method using dilute brain thromboplastin in mixtures of plasma fractions prepared by ammonium sulphate precipitation from normal and haemophilic plasma. In the curve marked ○ the fibrinogen, globulin, and albumin were derived from haemophilic plasma. In the curve marked × the normal fibrinogen fraction was mixed with globulin and albumin from haemophilic plasma. In the curve marked ● the haemophilic globulin fraction was mixed with fibrinogen and albumin from normal plasma.

Discussion

From the foregoing experiments it would seem that the defect in the clotting mechanism of haemophilic blood is associated with a failure to react normally to small amounts of thromboplastin. There is no evidence that there is a deficiency of thromboplastin in haemophilic blood, compared with normal, nor of any abnormal inhibition or destruction of thromboplastin. Since the so-called antihaemophilic factor largely corrected the defective response of haemophilic plasma to thromboplastin, it appears that this factor is concerned in some way in potentiating or accelerating thromboplastic action.

The antihaemophilic factor is not essential for the thromboplastic action of brain, since strong suspensions of thromboplastin will clot haemophilic blood as readily as normal blood, but its accelerating effect becomes marked as the concentration of thromboplastin is reduced.

The mode of action of the antihaemophilic factor is difficult to investigate without fractionation of the plasma, yet this fractionation itself introduces serious difficulties. In the first place simple methods of fractionation do not always give a clear-cut separation, even of such well-recognized plasma constituents as fibrinogen and antithrombin. One experiment is never exactly comparable with another. In addition it is never possible to be certain that fractionation does not separate artificially components which, in the body, react as one physiological unit. Conclusions drawn from fractionation experiments must always be made cautiously, and regarded only as provisional hypotheses until more reliable methods of verifying the results are available.

The fractionation experiments recorded in this study appear to show that the Factor V and prothrombin of haemophilic and normal plasma react to weak brain suspensions in the same manner. The accelerating effect of the antihaemophilic factor on the haemophilic fractions appears to depend on the reconstitution of a whole plasma containing the albumin fraction in addition to the other components.

It is probable that in natural conditions the coagulation of the blood is largely dependent on such intrinsic thromboplastin as it possesses. The platelets may provide some or all of this, since it has been shown (Biggs, 1951a) that they have definite thromboplastic activity, despite previous contrary views (Ware, Fahey, and Seegers, 1948). Measured in terms of brain thromboplastin this intrinsic thromboplastin is relatively feeble, being equivalent only to a 1/10,000 suspension of brain extract, and it is at such levels that the action of the antihaemophilic factor becomes of great importance. Under these conditions a deficiency of this factor will result in such a slow prothrombin conversion that the rate of thrombin generation barely exceeds the rate of its destruction, and the concentration required to react with fibrinogen may not be achieved for a considerable time, if at all.

Summary

When dilute brain thromboplastin was added to haemophilic and normal plasma the haemophilic plasma had a longer clotting time on recalcification than normal.

The abnormal reaction of haemophilic plasma to thromboplastin could not be corrected by the addition of thromboplastin.

Haemophilic plasma had no specific ability to destroy thromboplastin.

In the two-stage method, using dilute thromboplastin, haemophilic plasma caused the formation of thrombin more slowly than normal plasma.

When the globulin fractions of normal and haemophilic plasma were separated from their respective albumin fractions, and tested by the two-stage method, using dilute brain thromboplastin, the speed of thrombin formation was the same in the two fractions.

The slow speed of thrombin formation which characterized whole haemophilic plasma was obtained when the haemophilic albumin and globulin fractions were mixed. The haemophilic albumin fraction had no inhibitory effect on the normal globulin fraction.

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PROTHROMBIN CONSUMPTION IN HAEMOPHILIAC KINDRED

BY

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In 1911 Addis noted that patients with haemophilia are unable to use prothrombin. This observation has been extended by Milstone (1948) and Quick (1947) with the development by the latter of the prothrombin consumption test and its later modification (Quick and Favre-Gilly, 1949). It has been hoped that this test would prove a diagnostic aid in the recognition of haemophilia, especially in those cases where the clotting time is within normal limits or only slightly prolonged. Recently such a case came under observation at the Toronto General Hospital.

Case Report

Victor B., a 19-year-old French Canadian boy, had three molar teeth extracted on December 21, 1949. Bleeding occurred from the sockets, and despite local measures, such as suturing the gums, the application of topical thrombin, and of local adrenaline, it continued for the next two weeks. He was admitted to the Toronto General Hospital on January 4, 1950. At this time the haemoglobin was 68% (10.6 g.) and the clotting time was 14 minutes by the modified Lee and White method, which was considered to be at the upper limit of normal. A prothrombin consumption test showed moderate but definite impairment of prothrombin utilization. A diagnosis of haemophilia was made. It was learned from a brother that abnormal bleeding had occurred in several male members of the family.

During the next eight days the patient received 6,000 ml. of fresh dextrated blood with an immediate decrease in the severity of the bleeding. After the last transfusion slight oozing persisted for another week. During the transfusions of fresh blood the clotting time and prothrombin consumption were found to be normal, but quickly reverted to their former levels when therapy was stopped.

Several years before the patient had experienced a similar episode of uncontrollable bleeding after tooth extraction which necessitated admission to another hospital for transfusions. The patient did not see fit to divulge this information until towards the end of his stay in hospital. There was no history of joint pain, epistaxis, haematuria, or bleeding from the bowel. He stated that minor wounds bled profusely.

The patient belonged to a prolific family living in the vicinity of North Bay, Ontario. It was decided to conduct a field trip to investigate (1) the history of bleeding; (2) clotting time; (3) prothrombin consumption three hours after the blood had clotted; and (4) hereditary characteristics including colour vision, taste blindness to phenyl-thio-carbamide, eye colours, tongue curling, blood groups A, B, O, M, N, and common Rh sub-types.

Procedure

Clotting time was measured at room temperature (approximately 15° C.). One millilitre of blood was placed in a small test-tube measuring 10 mm. in internal diameter. The tube was tilted every 30 seconds and the end-point was taken when gentle tapping would no longer cause the blood to flow.

Prothrombin consumption was measured by allowing approximately 5 ml. of venous blood to clot. After one and one half hours the clotted blood was centrifuged. At three hours the prothrombin time of the serum was measured by the addition of 0.1 ml. of serum to 0.1 portions of Seitz-filtered human plasma, thromboplastin (Difco), and 0.025 molar calcium chloride solution. The mixture was placed in a water bath at 37° C. The appearance of clot constituted the end-point The Seitz-filtered plasma provided a source of fibrinogen. It had been stored for a week, and on testing no prothrombin activity was observed. A short prothrombin consumption time indicated an impairment in prothrombin utilization such as is found in haemophilia.

Results and Discussion

The co-operation of the family was extremely satisfactory, as 85 members attended (Fig. 1). Twelve of the males had definite history of bleeding. Nine of these are still alive. The disease seemed to be uniformly mild in this family, as only one instance of haemarthrosis was noted, that in Rene B., the patient's brother, who later bled to death following a tonsillectomy.

The results of the prothrombin consumption test and the clotting times are presented in Table I. The mean clotting time of the bleeders was significantly higher than that of either the unaffected males or females. Similarly the prothrombin

TABLE I

COMPARISON OF HAEMOPHILIAC AND NORMAL GROUPS WITH REGARD TO PROTHROMBIN CONSUMPTION TIME, CLOTTING TIME, AND AGE

Factor	Group	No.	Mean	S.D.	Difference in Means
Prothrombin consumption time (sec.)	Haemophiliacs	8	37·3	13·4	Females and haemophiliacs. Diff. = 8·4.‡ t = 1·58
	Females	27	45·7	13·2	Unaffected males and haemophiliacs. Diff. = 16·9.* t = 3·28
	Unaffected males	32	54·2	12·9	Unaffected males and females. Diff. = 8·5.† t = 2·49
Clotting time .. (min.)	Haemophiliacs	7	19·1	5·14	Females and haemophiliacs. Diff. = 3·4.† t = 2·05
	Females	22	15·7	3·37	Unaffected males and haemophiliacs. Diff. = 5·2.* t = 3·61
	Unaffected males	29	13·9	2·98	Unaffected males and females. Diff. = 1·8.† t = 2·02
Age	Haemophiliacs	8	13·1	5·75	Females and haemophiliacs. Diff. = 5·1.‡ t = 1·23
	Females	27	18·2	11·3	Unaffected males and haemophiliacs. Diff. = 7·2.‡ t = 1·15
	Unaffected males	32	20·3	17·4	Unaffected males and females. Diff. = 2·1.‡ t = 0·54

* Difference is statistically significant at the 0·01 level of confidence.
† Difference is statistically significant at the 0·05 level of confidence.
‡ Difference is not statistically significant.

consumption time of the bleeders differed significantly from the unaffected groups, the mean of the former being lower. Thus it was possible by means of either of the two tests to differentiate on a group basis between haemophiliac males and unaffected members. However, due to the number of haemophiliacs having prothrombin consumption times and clotting times within the normal range, these tests were not considered sufficiently reliable for individual diagnosis.

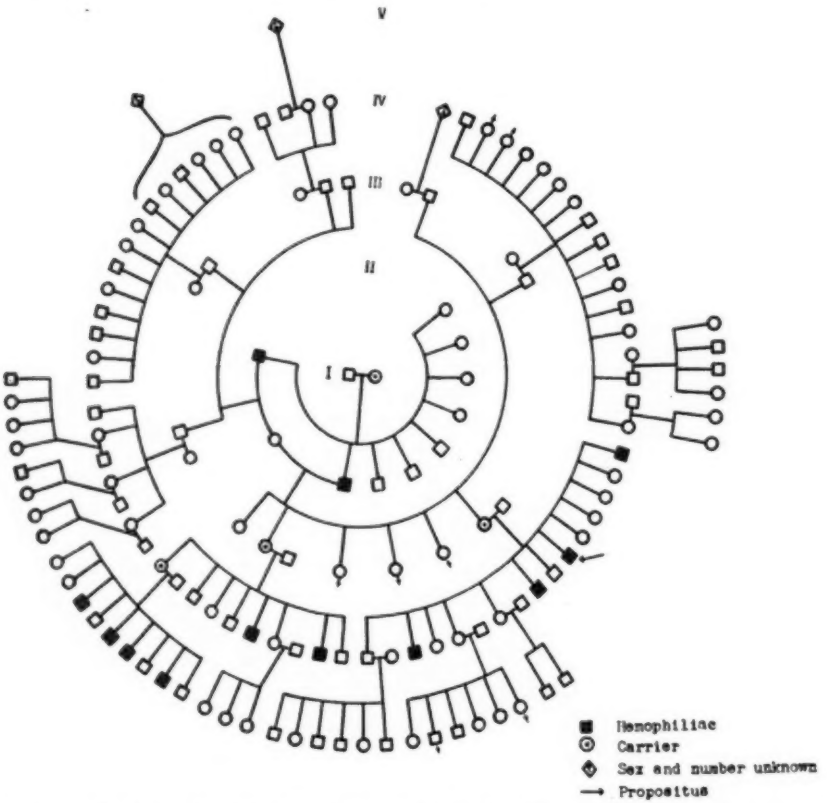

FIG. 1.—Pedigree showing five generations. The propositus (Victor B.) is indicated by arrow in the fourth generation.

Quick (1951) believes the prothrombin consumption test to be a valuable new means of establishing a diagnosis of haemophilia, and allows the diagnosis to be made even in the atypical case and before any abnormal bleeding has occurred. It must be emphasized that the present survey was carried out in the field under rather primitive conditions, and it is possible that different results might have been obtained in the laboratory, where a more complete investigation of prothrombin consumption

could have been carried out by means of methods described by Quick (1947) and Merskey (1950).

The mean prothrombin consumption time for three affected males, aged 19–21, was 29, while that of five affected, aged 6 to 12, was 42. Also, only 9% of the normal group had times shorter than 37 seconds, the longest time obtained for the older group. This suggests that in children the prothrombin consumption test is less helpful than in adults. If prothrombin consumption time depends on age, unsatisfactory results would be obtained by comparing haemophiliacs with normals of a different age range. In the present study, the mean age (see Table I) of haemophiliacs was lower than the mean ages of the unaffected groups. These differences in ages, however, were not statistically significant. Within unaffected males, there was no significant correlation (product moment $r = -0.241$) between age and prothrombin consumption time. Furthermore, due to the smallness of the haemophiliac group the results are only suggestive of a possible relationship in haemophilia between age and prothrombin consumption time.

Two incidental findings, which may be significant in another context, are presented in Table I. First, the prothrombin consumption time of the females tended to be lower than that of unaffected males. Secondly, the clotting time of the females tended to be longer than that of unaffected males.

Data concerning the hereditary characteristics were obtained for two reasons. Colour vision was investigated in the hope of adding to previous data on the sex-linkage of haemophilia and colour blindness. All the individuals examined, however, had normal colour vision. In the event of identifying different degrees of severity of the disease, the autosomally inherited characteristics were studied for their possible genetic linkage with a modifying factor. As the condition was found to be uniformly mild, this linkage study was not carried out.

Summary

Eighty-five members of haemophiliac kindred were investigated by means of a field survey. The disease was uniformly mild, and under the conditions of the present study the clotting time and prothrombin consumption are of limited value in diagnosis in the individual case. Without a history of excessive bleeding a diagnosis of haemophilia would not have been made with any degree of certainty. It was possible by these tests to differentiate, only on a group basis, haemophiliac males from unaffected members.

The funds for this investigation were provided by the Banting Research Foundation. We should like to thank Dr. K. J. R. Wightman for his interest and co-operation; Mr. A. D. McClure, Director, Provincial Laboratory, North Bay, for his assistance; and the remainder of the medical and technical team for their enthusiastic support.

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THE SLANTED CAPILLARY METHOD OF RHESUS BLOOD-GROUPING

BY

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In 1944 a method of Rh blood-grouping, using slanted capillary tubes, was described (Chown, 1944). Further experience with the method led to the appreciation of certain errors in it, which, in 1946, we pointed out, together with ways of preventing them (Chown and Lewis, 1946). Although Discombe and Meyer (1948) reported favourably on the revised method, descriptions of the original one remained more readily available in Britain (Mollison, Mourant, and Race, 1948; Pickles, 1949; Poole and Williams, 1951), so that, because of the inherent errors in this form, the method in any form fell into disrepute.

We have used the revised method on many thousands of blood samples, and believe that, properly carried out, it is simple, rapid, accurate, and economical for Rh-grouping. The slanted capillary has further advantages and uses which we shall report on later.

Accuracy of the Method

Since we do not do Rh-grouping by any test-tube method we can offer, as proof of the accuracy of our method, only comparisons with the results of others who do. The following three studies are so offered.

In 1949, as part of a study of the Rh chromosome distribution of the white population of the Province of Manitoba, we tested, with anti-C, anti-D, anti-E, and anti-c, 792 unselected individuals of English descent (Chown, Peterson, Lewis, and Hall, 1949). Our results are compared in Table I with those of Race, Mourant, Lawler, and Sanger (1948), who tested 2,000 English people by their test-tube method. (A test for homogeneity gives $\chi^2=7.711$; d.f.$=9$; $P=0.5$.) The two series do not differ significantly in distribution of Rh phenotypes. From this we deduce that the two methods of testing give closely comparable results.

In 1949 Dr. Philip Levine, of the Ortho Research Foundation, generously gave us some of his anti-k serum "Cellano." With this serum Levine, Backer, Wigod, and Ponder had tested 2,500 unselected white Americans. With it we (Chown, Lewis, and Peterson, 1951) tested 2,872 unselected white Canadians. The results are compared in Table II ($\chi^2=0.1108$; d.f.$=1$; $P=0.07$). Again the results do not differ significantly, so that we deduce that the two methods give comparable results.

Dunsford (1949) and Bertinshaw, Lawler, Holt, Kirman, and Race (1950) tested 1,041 English people with Dunsford's anti-K serum "And." We tested the above

TABLE I

RESULTS OF RH TEST IN ENGLAND BY TEST-TUBE METHOD AND IN CANADA BY THE CAPILLARY METHOD

Reactions to Anti-sera C, D, E, c	Rh Phenotype	English (Race *et al.*)		Canadians of English Origin	
		Absolute Numbers	%	Absolute Numbers	%
+ + − +	R_1r	689	34·45	256	33·45
+ + − −	R_1	373	18·65	140	17·68
− − − +	r	307	15·35	134	16·92
− + + +	R_2	279	13·95	117	14·77
+ + + +	R_1R_2	270	13·50	107	13·51
− + − +	R_0	42	2·10	13	1·64
− − + +	R^y	19	0·95	7	0·88
+ − − +	R′r	16	0·80	6	0·75
+ + + −	R_z	5	0·25	1	0·13
+ − − −	R′	0	0	2	0·25
Total ..		2,000		783	

TABLE II

RESULTS OF TESTS WITH ANTI-CELLANO SERUM IN THE UNITED STATES BY THE TEST-TUBE METHOD AND IN CANADA BY THE CAPILLARY METHOD

Reactions to Anti-Cellano	Americans (Levine *et al.*)	Canadians
+	2,495	2,865
−	5	7

2,872 white Canadians with our anti-K serum "Ram." The results are compared in Table III ($\chi^2 = 0.0543$; d.f. = 1 ; P = 0.8). Again, since the results do not differ significantly, we deduce that the two methods give comparable results.

TABLE III

RESULTS OF TESTS WITH ANTI-K (AND) BY THE TEST-TUBE AND WITH ANTI-K (RAM) BY THE CAPILLARY METHODS

Reactions to Anti-K	"And" (Bertinshaw *et al.* and Dunsford)	"Ram"
+	74	198
−	967	2,674

Method

Apparatus.—The necessary apparatus is readily found in any laboratory and is not expensive.

Sera.—The sera must agglutinate cells in saline suspension. They must be free of coagula. While weak sera may be used, it is better to use an avid serum with a titre of

32 or more, and dilute it with saline to a titre of 8. Anti-A and B we neutralize by Witebsky's A and B substance.*

Capillary Tubes.—We use capillary tubes such as are sometimes used to determine clotting time. They are approximately 3½ in. long, with a bore of roughly 0.4 mm.† These we buy in bulk, 15 lb. at a time. A capillary is used only once and thrown away.

Agglutination Stand.—What is needed is something to seal the lower ends of the capillaries and to hold them at an angle of 45 degrees. We use a home-made holder, costing about $1, made of thin building-board, in the form of a box 15 in. long × 4 wide × 4 high (Fig. 1). The lights in the box are for illumination, not for warmth. The plasticine along the lower edge of the opal glass is to seal and hold the capillaries. Discombe and Meyer (1948) also describe a simple apparatus.

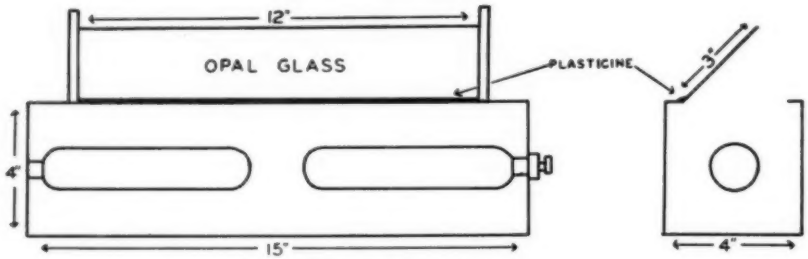

FIG. 1.—Diagram of agglutination stand.

Preparation of Cells.—For single tests—for example, at the bedside—or for emergency Rh-typing when transfusion is urgently required, the finger is pricked and a drop of blood expressed. A drop of citrate or oxalate, two or three times its size, is placed beside it, and mixed.

For routine laboratory use, and particularly when large numbers of samples are to be tested, oxalated, citrated, or heparinized blood or cells freed from clotted blood may be used. One or two large drops of blood are dropped into a small test-tube (say, 1 × 9 cm.: it must be shorter than the capillaries), and filled with saline, using *at least* 10 volumes of saline to 1 of blood. The mixture is centrifuged briefly to throw down most of the cells but not to pack them, and the saline is poured off quickly. We have developed a quick flip at the end of the pour that gives just the right suspension. It is a matter of practice and of habit, although there is some latitude as to the final suspension that will give sharp results, say 20 to 40%. If saline-diluted serum is to be used the red cells need no further treatment: the drop or two of suspension in the bottom of the tube is what you want. *If whole serum is to be used repeat the washing twice.*

Loading and Setting up the Capillary Tubes.—The agglutinating serum should be in a container less deep than the length of the capillaries—for example, a small test-tube or bottle. The tip of the capillary is dipped in the serum and ½ to 1 in. of serum allowed to run in. The capillary is placed on the table on a gentle slope, serum-filled end down. By so doing you prevent air from following the serum in. The process is repeated until you have six to 10 such serum-containing capillaries lying on a slope, parallel to one another,

* Kindly supplied by Sharp and Dohme (Canada) Limited, 1100 O'Connor Drive, Toronto 13, Ontario.

† Baird and Tatlock have sent us sample capillary tubes which were quite satisfactory.

but a little apart. Now the first capillary is picked up in one hand and the test-tube containing the first red cell suspension in the other. With the latter on a fairly long slant, the serum-filled end of the capillary is dipped into the red cell suspension and an amount of the suspension roughly equal to the serum is allowed to run in. The capillary tube is turned red-cell-end up and the other is stuck in the plasticine with the capillary lying against the glass. The procedure is repeated with the next capillary and the next blood, and so on.

For a single test serum is taken up in the same way and then the serum-filled end is dipped into the mixed oxalated blood. The capillary is turned red-cell-end up and the other end is stuck at an angle of 45 degrees in a lump of plasticine. This is carried back to the laboratory along with such other specimens as you may be taking—for example, blood for haemoglobin, ABO-grouping, etc. There is a good chance that you can read your Rh-grouping by the time you get back to the laboratory.

The Reaction.—Rh-positive cells set up against a saline-active Rh serum form into clumps, at first fine, then more or less quickly enlarging and appearing as cross bars with rather clear spaces in between. Eventually, the clumps will slide down towards the butt of the tube, and one will have left a solid butt with clear serum and saline above. Rh-negative cells form no clumps, but instead a long narrow ribbon or needle of cells along the back wall of the capillary, the ribbon or needle being thinner and more pointed above. The Rh-negative cells will stay thus for hours.

We read our Rh-positive reactions as soon as clumping is clearly visible, usually, with a good serum, within five to 10 minutes. Some cells react slowly with some sera, so that we do not make a final reading of Rh-negative until the end of an hour. You soon get to know your sera.

Some Minor Points.—If an air bubble gets locked between the serum and the cells, blow the contents of the capillary out on your thumb-nail, and then let the mixture run back into the capillary, and set it on the stand as usual. With experience these bubbles hardly ever occur.

If several sera—for example, anti-C, anti-D, anti-E, etc.—are to be used with one lot of cells, the cells may become contaminated by the earlier sera unless the following simple dodge is used : as you pick the capillary up in your right hand run the serum-filled end between the thumb and forefinger of your left to wipe off serum on the surface. After a time this motion will become automatic.

Do not keep the light on or it will make the glass too warm. Turn it on only to read the reactions

The method works well with blood several days old, provided it is freshly washed before testing.

Errors in the Method

False Positives.—These may be due to technical errors or to unrecognized antibodies.

Due to Technical Errors.—There will be no false positives if the directions are followed. *They are practically all due to not washing the cells ;* once, if a diluted serum is to be used ; thrice, if undiluted serum is to be used. Too high a concentration of serum or plasma in the capillaries may lead to clumping indistinguishable to the inexperienced from true agglutination. This is perhaps comparable to or identical with rouleaux formation. It can occur with some bloods if one simply sets a patient's unwashed cells up with his own serum.

Due to Unrecognized Antibodies.—The test is so sensitive that traces of antibodies will occasionally give reactions by this method when they do not evidence themselves by the test-tube method. This has been true, for example, of specimens of anti-C antibody derived from anti-C + D by absorption of the anti-D. If this possibility is borne in mind new sera will be tested against appropriate panels of cells and, if necessary, further absorbed.

False Negatives.—If one is using a good serum, real false negatives never occur. If, however, one uses too dilute a suspension of cells, visible clumping will be delayed and the clumps will be so fine that they are not easy to see with the unaided eye. The error is readily avoided by not using dilute suspensions. If only a few cells are available for testing, one may get around the difficulty in several ways : removal of the saline from the washed cells by means of a fine capillary ; using a shorter column of serum and cells ; laying the tube down flat on the table for 15 minutes after the cell-serum mixture is in it, before putting it up on the slant.

Advantages of the Method

Economy of Serum in Any Laboratory.—About 100 tests can be done with 1 ml. of serum. If the serum is avid it may be diluted to give several hundred tests per ml.

Economy of Man-power in Public Health Laboratories.—For nearly four years Rh-typing and search for and titration of antibodies have been offered as a free public health service for all pregnant women in Manitoba. Using the capillary method for Rh-typing and for discovery and titration of saline antibodies, and the short modification (Chown, Lewis, and Bryce, 1948) of the Diamond-Denton method for albumin antibodies, one technician can do all routine pregnancy testing for a population of 1,000,000. This includes, in addition to Rh-typing of all pregnant women, and of the husbands of the Rh-negative women, phenotyping of all the family of pregnant sensitized women, as well as periodic titration of maternal antibody.

Economy of Time in the Clinical Laboratory.—Rh-typing is commonly done in connexion with transfusions, pregnancy, or disease in newborn babies. Ordinarily the typing may be done with deliberation. Upon occasion speed is important. As indicated above, most Rh-positive bloods will declare themselves in five to 10 minutes, while, by using the single test, bedside method, Rh-typing can often be completed by the time one has set up his ABO-grouping.

Economy of Methods.—We have found the method useful with antisera of the following specificities : D, C, C^w, c ; E, e ; K, k ; S ; Lu^a ; Jk^a ; Le^a. Some sera, that others have reported to agglutinate only albumin-suspended cells or contain antibodies demonstrable only by the indirect Coombs technique, we have found to agglutinate saline-suspended cells by the capillary method. This allows a saving of time, temper, money, and rare sera.

Summary

The slanted capillary method in our hands has proved to be accurate. It is rapid in itself and, being exceedingly simple, allows the testing of many blood specimens a day. It is economical of serum. It may be used, not only with anti-Rh sera, but also with a number of others.

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THE QUESTION OF THE Rh HAPTEN

BY

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The claim of Carter (1949) to have isolated from D-positive red cells a hapten-like substance reacting specifically with anti-D sera aroused universal interest. From Rhesus-positive erythrocytes she extracted with ether a piloid substance soluble in alcohol. An emulsion in saline of this solution gave a positive complement fixation test with anti-D sera and also caused inhibition of such sera. She further reported the clinical use of this substance which, when injected, caused a fall in the Rh agglutinins in sensitized pregnant women. There has, however, been no confirmation of this work from any other source, though some have failed to substantiate the original claims (Coombs, 1950; Stratton and Renton, 1950). The practical value of such a substance seemed sufficiently obvious to justify an investigation of the problem if only to indicate some of the difficulties involved.

Landsteiner and von Eisler (1904) first showed that petroleum ether extracts of animal red cells had an inhibitory effect on haemolytic antisera prepared from the same species of cell. Balls and Korns (1918) failed to demonstrate any specific properties in the lipoids extracted from sheep cells.

Sachs and Klopstock (1925) first showed that such substances as lecithin were antigenic when injected together with a foreign protein. Van der Scheer, Levene, and Landsteiner (1927) were able to confirm this, using commercial ovo-lecithin. Landsteiner concluded that the positive results were due to the presence of impurities in the ovo-lecithin, and there have subsequently been many conflicting reports on the antigenicity of lecithin.

Landsteiner and van der Scheer (1925) obtained alcoholic extracts from red cells of various species, and their extracts corresponded fairly closely to the first filtrate in Carter's method. They found that horse blood extracts would not inhibit immune sera prepared against the whole blood, from which one may fairly conclude that such extracts do not necessarily contain all the antigenic components of the cells.

Belkin and Weiner (1944) showed that the red cell stroma possessed Rh specificity, and subsequently Calvin, Evans, Behrendt, and Calvin (1946) reported the existence of an ether-soluble fraction of the stroma which also had Rh specificity. This fraction they called elinin, and the work of these latter authors is the basis of the experiments reported by Carter.

Spielmann (1949) showed that alcoholic extracts purified by acetone precipitation would inhibit all antibodies of the Rh system, the effect being similar for anti-D, anti-C, and anti-c.

Progress in the isolation of the other blood group substances has been greatly facilitated by their occurrence in water-soluble form, and a considerable amount is known about them from a chemical point of view, but of the Rh antigens, so far unconfirmed in body fluids, we know nothing.

Materials and Methods

The Cell Extracts.—The method described by Carter (1949) was carefully followed. The "positive" extract was obtained from four D-positive donors, the genotypes being cDE/cde (3) and CDe/cDE (1). The "negative" extract was similarly obtained from four D-negative donors (cde/cde). All donors were group A. After ether extraction the solvent was removed by distillation under reduced pressure, the temperature never rising above 30° C., and the residue finally dried over phosphorus pentoxide with a high vacuum pump. A 1% solution in ethyl alcohol was made, and this appeared to be almost saturated at room temperature.

Inhibition Tests.—These were carried out by the following technique:

Doubling dilutions of serum were set up in two or more rows with saline controls. To the first row of tubes was added equal volumes of saline, and to the succeeding rows equal volumes of extract as indicated in the tables.

The results were recorded as follows:

C	= one to three large clumps
+++	= smaller macroscopical clumps
++	= microscopical, mainly large clumps
+	= clumps of a dozen or more cells with many loose ones
w	= clumps of two or three cells
—	= no agglutination

Complement Fixation Tests.—These tests were based on the method described by Boyd (1947). The following scheme was adopted:

Extract emulsion	0·1 ml.
Serum	0·05 ,,
G.P. complement (2½ units)	0·25 ,,
Saline to make total volume of	0·7 ,,

The tubes were allowed to stand at 2° C. for 18 hours and then for 10 minutes at 37° C. after which 0.3 ml. of haemolytic system (2 vols. of haemolytic serum containing 5 units to 1 vol. of 5% suspension of washed sheep cells) was added. After incubating for 30 minutes at 37° C. the tubes were centrifuged at 1,500 r.p.m. for 2 minutes and readings taken. Results were recorded as follows:

C	= complete haemolysis
ac	= almost complete haemolysis
+	= partial haemolysis
Tr	= trace of haemolysis
—	= no haemolysis

Results

The results are divided into two parts, the first recording Carter's preparation, whilst the second comprises a few further experiments which seemed relevant to the problem.

Part I.—Not surprisingly, the extracts were found to be haemolytic as indicated in Table I, and this placed limitations on the concentrations which could be used.

Inhibition tests were carried out with anti-D sera, using extracts in minimal non-haemolytic dilutions, and typical results are shown in Tables II and III.

TABLE I

HAEMOLYTIC* TITRES OF EXTRACTS IN SALINE

Extract	Temperature	Dilutions in Saline					Control
		2	4	8	16	32	
"Positive" ..	Room	C	—	—	—	—	—
	(37° C.)	C	C	—	—	—	—
"Negative" ..	Room	C	+	—	—	—	—
	(37° C.)	C	C	+	—	—	—

* Haemolysis of D-positive red cells.

TABLE II

INHIBITION TEST WITH "POSITIVE" EXTRACT AT ROOM TEMPERATURE*

Row	Dilutions of anti-D in Saline									Controls
	2	4	8	16	32	64	128	256	512	
1. Saline ..	C	C	C	†	+++	+	—	—	—	— —
2. Alcohol 1 : 4	C	C	C	+++	+++	w	—	—	—	— —
3. Extract 1 : 4	C	C	+++	+++	+++	+	— —	—	—	— —

* Agglutination of D-positive red cells. † Spoilt tube.

TABLE III

INHIBITION TEST WITH "NEGATIVE" EXTRACT AT ROOM TEMPERATURE*

Row	Dilutions of Anti-D in Saline									Controls
	2	4	8	16	32	64	128	256	512	
1. Saline ..	C	C	+++	+++	+++	+++	+	—	—	— —
2. Alcohol 1 : 4	C	C	+++	+++	+++	+	w	—	—	— —
3. Extract 1 : 5	C	C	+++	+++	+++	+	—	—	—	— —

* Agglutination of D-positive red cells.

It will be seen that in row 3 of Table II there is a slight loosening of the clumps, but there is no unequivocal inhibition, specific or otherwise.

In order to test higher concentrations of the extracts, dilutions of anti-D were set up in normal group AB serum, which inhibits the lytic effect. The result of such a test is shown in Table IV. It will be seen that there is relative inhibition by the "positive" extract, but it is of doubtful significance.

TABLE IV

INHIBITION TEST IN AB SERUM AT ROOM TEMPERATURE*

Row	Dilutions of Anti-D in AB Serum							Controls
	2	4	8	16	32	64	128	
1. "Positive" extract	+++	+++	+++	+		—	—	— —
2. "Negative" extract	+++	+++	+++	+++	+++	—	—	— —

* Agglutination of D-positive red cells.

Finally, dried extracts were tried: 0.05 ml. of the 1% alcoholic solution was evaporated to dryness in a tube with a vacuum pump. Then 0.05 ml. of anti-D serum was added and the mixture allowed to stand at room temperature for one hour. The serum was run out in serial dilution and titrated. Table V shows the comparison of the results obtained simultaneously with "positive" and "negative" extracts. Again, there is no significant difference.

TABLE V

INHIBITION TESTS WITH DRIED EXTRACTS AT ROOM TEMPERATURE*

Row	Dilutions in Saline										Controls
	1	2	4	8	16	32	64	128	256	512	
1. Anti-D† alone ..		+++	+++	+++	+++	+++	+++	w	–		––
2. Anti-D+ positive extract ..	C	C	C	C	+++	+++	+	w	–	–	––
3. Anti-D+ negative extract ..	C	C	C	+++	+++	+++	+++	–	–	–	––

* Agglutination of D-positive red cells.
† An independent titration of the same serum included for comparison of the final titre.

Complement Fixation.—It was found that the extracts were anticomplementary, a not uncommon feature of tissue extracts, and attempts to determine anticomplementary titres at low temperature gave inconsistent results. In a series of 20 titrations on the same extract, results were obtained varying from 1 : 500 to 1 : 64,000. The widest variations appeared to be associated with different pools of guinea-pig serum used for complement and were independent of the age of the complement, as is shown in Table VI, in which different complement pools are compared. The guinea-pig serum was stored at −20° C. when not in use.

TABLE VI

ANTICOMPLEMENTARY TITRES USING DIFFERENT COMPLEMENT POOLS INCUBATED AT 2° C. OVERNIGHT*

Complement Pool (2½ units)	Dilutions of Extract (in hundreds)										Complement Controls
	5	10	20	40	80	160	320	640	1,280	2,560	
No. 1 (fresh) ..	Tr	+	ac	ac	C	C	C	C	C	C	C
No. 1 (17 days)†	ac	ac	ac	C	ac‡	C	C	C	C	C	C
No. 2 (10 days)	–	–	–	Tr	Tr	+	C	C	C	C	C
No. 3 (6 days)	+	+	ac	C	C	C	C	C	C	C	C
No. 4 (fresh) ..	–	–	–	–	Tr	+	ac	C	C	C	C
Preserved§ ..	–	–	–	–	–	–	–	+	ac	C	C

* Haemolysis of sensitized sheep red cells. † Complement stored at −20° C. ‡ Probable impurity in tube.
§ "Wellcome" preserved complement (Richardson's method).

Using Pool No. 3, which gave the lowest anticomplementary titre, complement fixation tests were performed using the positive extract and an anti-D serum having a saline agglutinin titre of 1 : 128. The result was negative, as shown in Table VII.

TABLE VII

COMPLEMENT FIXATION TESTS WITH "POSITIVE" EXTRACT AND ANTI-D SERUM INCUBATED AT 2° C. OVERNIGHT*

Extract Dilution	Extract Controls		Dilutions of Serum					
			Anti-D				Normal Serum	
	Haemo-lytic	Anti-complementary	4	8	16	32	4	8
2,000	—	+	ac	+	+	+	ac	ac
4,000	—	ac	C	ac	ac	ac	C	C
8,000	—	C	C	C	C	C	C	C
16,000	—	C	C	C	C	C	C	C
32,000	—	C	C	C	C	C	C	C
Serum controls	Anticomplementary		C	C	C	C	C	C
	Haemolytic		—	—	—	—	—	—

* Haemolysis of sensitized sheep red cells.

Part II.—The conclusion to be drawn from these results is in harmony with the statements made by Coombs and Stratton.

In the preliminary tests, however, following the normal procedure wi h Rh antibodies, the inhibition tests were carried out at 37° C., and many irregular and inconsistent results were obtained in the form of haemolysis and partial inhibition. These appeared to be due to the effect of the alcohol on the serum.

When alcohol was incubated with normal serum at 37° C. for periods greater than one hour, the product became increasingly haemolytic, producing complete haemolysis at the end of two and a half hours' incubation. The effect was readily produced with 50% alcohol, and lower concentrations were also effective, but the effect of alcohol on different anti-D sera was very variable.

As no lysis occurred at room temperature the inhibition tests recorded were carried out at room temperature. It seemed probable that as a preliminary to lysis there was some loosening of the clumps which might simulate inhibition, and the variability of the effect might well give false impressions. However, the results obtained at room temperature were at least consistent.

Extracts with organic solvents inevitably contain much lipoid material, and this group of substances is known to give rise to non-specific effects. Spielmann (1949) showed that purified ovo-lecithin would inhibit antibodies of the Rh system just like his purified alcholic extracts of red cells, but would not apparently inhibit anti-A or anti-B.

Table VIII shows the results of inhibition tests with commercial ovo-lecithin and anti-sera of the ABO and Rh systems. A 1% solution of the ovo-lecithin in ethyl alcohol was made and diluted with saline to 1 : 40 (the lowest non-haemolytic dilution).

The preparation, which was used as received from the manufacturers without further purification, gave results somewhat at variance with those recorded by Spielmann. Inhibition is definite with the anti-A and anti-B, but slight with only one of the anti-D sera tested. The explanation may lie in the fact that Spielmann

TABLE VIII

INHIBITION TESTS WITH COMMERCIAL OVO-LECITHIN PERFORMED AT ROOM TEMPERATURE*

Serum	Equal Vols. of	Dilutions of Sera										Controls
		1	2	4	8	16	32	64	128	256	512	
Anti-A	Saline Lecithin (1 : 4,000)	C	C	C	C	C	+++	+	w	–	–	– –
		C	C	C	C	+	–	–	–	–	–	– –
Anti-B	Saline Lecithin (1 : 4,000)	C	C	C	C	+++	+++	+++	+	w	–	– –
		C	C	C	+++	+++	+	–	–	–	–	– –
Anti-D	Alcohol (1 : 40)			+++	+++	+++	+++	+	–	–	–	– –
	Lecithin (1 : 4,000)			+++	+++	+	w	–	–	–	–	– –
Anti-D	Saline Lecithin (1 : 4,000)		+++	+++	w	–	–	–	–	–	–	– –
			+++	+++	–	–	–	–	–	–	–	– –

* Agglutination of A, B, or D-positive red cells.

used a purer form of a substance which, in any case, appears to be unstable and deteriorates rapidly. In this connexion it should be mentioned that the first extract obtained from D-positive cells did produce a marked inhibition of one anti-D serum only (Table IX). It could never be repeated with other sera, and unfortunately that particular serum was no longer available later to be tested with a "negative" extract.

TABLE IX

INHIBITION TEST WITH "POSITIVE" EXTRACT AND AN ANTI-D SERUM PERFORMED AT 37° C.

Row	Dilutions of Anti-D										Control
	4	8	16	32	64	128	256	512	1024	2048	
1. Saline ..	+++	+++	+++	+++	+++	+++	+	w	–	–	–
2. 50% alcohol	+++	+++	+++	+++	+++	w	+	+	–	–	–
3. Extract ..	++	–	–	–	–	–	–	–	–	–	–

With regard to complement fixation it was thought possible that the actual technique of preparing the saline emulsion might influence the anticomplementary effect. However, a comparison of different methods of preparation (including allowing it to stand for two hours before use) revealed no difference. It was concluded, therefore, that the cause of the variation in anticomplementary effect of the extract was inherent in the material itself, especially since stabilized antigens do not give such remarkable variations in anticomplementary titres.

In view of the work of Landsteiner and van der Scheer (1925) on flocculation tests with alcoholic extracts, it was thought pertinent to investigate the activity of such extracts with anti-D sera. Extracts were prepared according to the original method of these workers, and the results are recorded in Table X. A dilution of 1 : 3 in saline was non-haemolytic. The result is seen not to be really significant, although there is a slight bias in favour of the "negative" extract.

TABLE X

INHIBITION TESTS WITH ALCOHOLIC EXTRACTS AND ANTI-D PERFORMED AT ROOM TEMPERATURE

Row	Dilutions of Anti-D								Controls
	2	4	8	16	32	64	128	256	
1. Saline	+++	+++	+++	+++	+++	+++	w	–	– –
2. Alcohol 1 : 3 ..	+++	+++	+++	+++	+++	+	–	–	– –
3. " Negative " extract 1 : 3 ..	+++	+++	+++	+++	+	w	–	–	– –
4. " Positive " extract 1 : 3 ..	+++	+++	+++	+++	+++	w	–	–	– –

Discussion

The well-recognized non-specific effects of lipoid substances clearly render the use of a " negative " control vitally essential, but even without this the results of inhibition tests in this work are mainly without significance. Moreover, the difficulties of working with alcoholic solutions seem to make the whole method very uncertain.

In complement fixation we are dealing with a much more complicated and much less understood mechanism, and it seems doubtful whether it possesses any advantages over inhibition tests with regard to sensitivity. We do not know whether or in what circumstances the Rh system is capable of fixing complement. During the present investigation attempts to demonstrate complement fixation with whole D-positive cells and an anti-D serum at 37° C. ended in failure. It probably resembles the ABO system which Schiff and Adelsberger (1924) showed to fix complement very poorly if at all, although Ervin and Young (1950) claim to have obtained complement fixation with an immune anti-A.

In the present investigation, the prime difficulty in complement fixation was the anticomplementary effect of the extract. Even with a suitable complement, the usable dilution of extract (1 : 4,000) was probably high enough for any specific activity to be lost. The uncertain behaviour of the extract with complement demands great caution in the interpretation of such tests, and the possibility of summation of anticomplementary effects of antigen and antibody (Eagle, 1932) must also be taken into account.

That red cell extracts can display inhibitory properties cannot be denied, but they are not necessarily specific in character. Spielmann's more dramatic results with alcoholic extracts are presumably related to the longer period of extraction (eight days as opposed to 24 hours in the method used here), and it is significant that his purified preparation was less active than the crude product.

It is conceivable that the presence of lipoid material in the extract may inhibit or otherwise mask its specific activity in *in vitro* tests. Spielmann produced a titre of antibodies in guinea-pigs by injecting group O, D-positive cells and demonstrated a fall in titre following the injection of his purified preparation of lecithin. The fall in titre of Rh antibody in two pregnant women observed by Carter need not be specific in the light of Spielmann's experiment, and it would be interesting to know

what effect the injection of lecithin would have on the titres of various other antibodies.

The experiments of other workers and those presented here confirm that lipoid substances give rise to considerable difficulties in serological work. In particular, lecithin has given very variable results in the hands of different workers. A similar state of affairs appears to exist in respect of the present problem, as is shown by the work of Spielmann and of the present author. Until these discrepancies can be satisfactorily controlled, it seems premature to talk of the Rh hapten.

Summary

Carter's (1949) method for the extraction of the so-called hapten from Rh-positive red cells has been carefully followed, and the products obtained tested for activity and Rh specificity by means of inhibition and complement fixation tests.

No significant inhibition with the final ether extract, specific or otherwise, could be demonstrated with the majority of anti-D sera used.

Haemolytic and inhibitory effects of alcohol are discussed in relation to the difficulties of performing such tests with alcoholic solutions.

Inhibition tests were also carried out with alcoholic extracts of D-positive and D-negative cells, and again no significant inhibition could be demonstrated.

Commercial ovo-lecithin was shown to have inhibitory properties with antisera of both the Rh and ABO systems.

Complement fixation tests were carried out with D-positive cell extracts at 2° C. overnight. No fixation could be demonstrated. The chief difficulty with these tests was the anticomplementary effect of the extract, which showed marked variation according to the complement used.

It is emphasized that lipoid substances are prone to give non-specific reactions and that the isolation of the Rh hapten has not been established on the published evidence.

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THE ACTION OF *p*-AMINOSALICYLIC ACID ON PROTHROMBIN TIME IN MAN

BY

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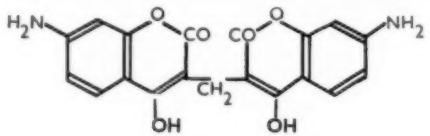
A lengthening in prothrombin time by giving salicylates was first observed in the animal experiments of Link, Overman, Sullivan, Huebner, and Scheel (1943). This result, and a comparison of the structures of coumarin-like anticoagulants (Overman, Stahmann, Huebner, Sullivan, Spero, Doherty, Ikawa, Graf, Roseman, and Link, 1944), led Link to conclude that dicoumarol (3,3′-methylenebis [4-hydroxycoumarin]) acts on breaking down *in vivo* to the chemically related salicylic acid. The experimental findings of Link and his colleagues have been amply confirmed and extended to man (Meyer and Howard, 1943 ; Shapiro, Redish, and Campbell, 1943 ; Clausen and Jager, 1946 ; Dibenedetto Dell' Aquila and Lojacono, 1946 ; Owen and Bradford, 1946 ; Zimmerman and Shapiro, 1946 ; Nitshe, Gerarde, and Deutsch, 1947). Their hypothesis, however, has not found complete acceptance, although the only direct evidence against it is the absence of salicylates in the urine after treatment with dicoumarol (Lester, 1944).

Jaques and Lepp (1947) found that rabbits given salicylate by mouth, or dicoumarol either by mouth or intravenously, showed a lengthening of prothrombin time. Intravenous salicylate had no effect, neither did salicylate given orally together with succinylsulphathiazole. They advanced the view that salicylate may be converted to a dicoumarol-like substance by bacterial synthesis in the intestine.

It was hoped that a comparison of blood salicylate levels and prothrombin times in patients receiving salicylate and in those receiving dicoumarol would yield further information on their mode of action. During this investigation, which was making slow progress owing to the lack of suitable patients, the analogous case of *p*-aminosalicylic acid (P.A.S.), used in the treatment of tuberculosis, was given consideration.



The structural relationship of P.A.S. to the dicoumarol analogue 3,3′-methylenebis [7-amino-4-hydroxycoumarin] is that of salicylic acid to dicoumarol itself, and their biological relationship may be expected to be similar. Thus on Link's hypothesis the dicoumarol analogue should break down to P.A.S. *in vivo* and give a more constant blood level of the drug than does the administration of P.A.S. itself. On the Jaques and Lepp theory the diaminodicoumarol or a related compound

may be synthesized *in vivo* from P.A.S., and its possible anticoagulant properties will assume importance. Attempts to synthesize diaminodicoumarol and related substances were made at our request by workers in the Research Department of Herts Pharmaceuticals Ltd. (Drain and Seymour).

The possible salicylate-like, antiprothrombin effect of P.A.S. has been noted by several observers. It was first mentioned as a possibility by O'Connor (1948), and some decrease in prothrombin levels was found by Bavin (1949) in animal experiments. Similar observations have since been reported by Nagley (1949), Swanson (1949), and Fisher (1949) in man. The blood prothrombin contents in these cases did not fall to danger level, but Fisher suggested that combined treatment with P.A.S. and streptomycin would increase the tendency towards hypoprothrombinaemia, since the bacteriostatic effect of streptomycin on *Esch. coli* might deplete the host of vitamin K. Barclay (1949) has reported an antiprothrombin effect of intravenous P.A.S. in man ; this resembles the findings of Link *et al.* (1943) and would seem to differ from those of Jaques and Lepp (1947). The most recent work on the subject is that reported from Farnborough Hospital by Madigan, Griffiths, Lynch, Bruce, Kay, and Brownlee (1950) and Lynch (1950). They have examined the question with the clinical aspect in mind, and think that the changes are probably not significant. Lynch assumes the analogy of P.A.S. to salicylate and discusses Link's hypothesis, which he finds is not confirmed. The same assumption is made here, and the theories of Link *et al.* and of Jaques and Lepp are assumed to refer equally to salicylic acid and to P.A.S.

Experimental

The investigation was conducted during the routine therapy of tuberculous patients and was subordinated to their course of treatment.

Clinical Material.—Three groups of patients were investigated.

Cases on P.A.S. Alone.—These were Cases 1–6.

Cases on P.A.S. and Streptomycin.—This group comprise Cases 7–9. Streptomycin was given with P.A.S. after the antiprothrombin effect of the latter alone had been observed. The effect of streptomycin alone was investigated in two control cases, patients suffering from tuberculosis.

Cases on P.A.S. and Succinylsulphathiazole.—These were Cases 12–14. P.A.S. therapy was supplemented in this group after a few weeks by a short course of succinylsulphathiazole. The effect of succinylsulphathiazole alone was investigated in three healthy volunteers (Cases 15–17, Table I). Case 17 was in addition given vitamin-K analogue " synkavit " (Roche).

Dosage.—Two schemes of P.A.S. dosage were used : 140 g. Na–P.A.S. per week (Cases 1, 5, 7, and 8), and 120 g. Na–P.A.S. per week (Cases 2, 3, 4, 6, 9, 10, 12, 13, and 14). The drug was given on six days, and not on the seventh of each week.

The dosage of streptomycin was 0.5 g. b.d. intramuscularly.

The " synkavit " given orally to Case 17 consisted of 10 mg. t.d.s. for two days before his control curve began to be charted ; it was then increased to 40 mg. t.d.s., and succinylsulphathiazole administration was started.

Methods.—Venous blood was collected before the beginning of P.A.S. therapy and thereafter usually at weekly intervals. Specimens were taken exactly 15 minutes (in Cases 1, 2, 3, 5, 7, 8, and 10) or exactly 30 minutes (in Cases 4, 6, 9, 12, 13, and 14) after the second (mid-morning) daily dose of P.A.S.

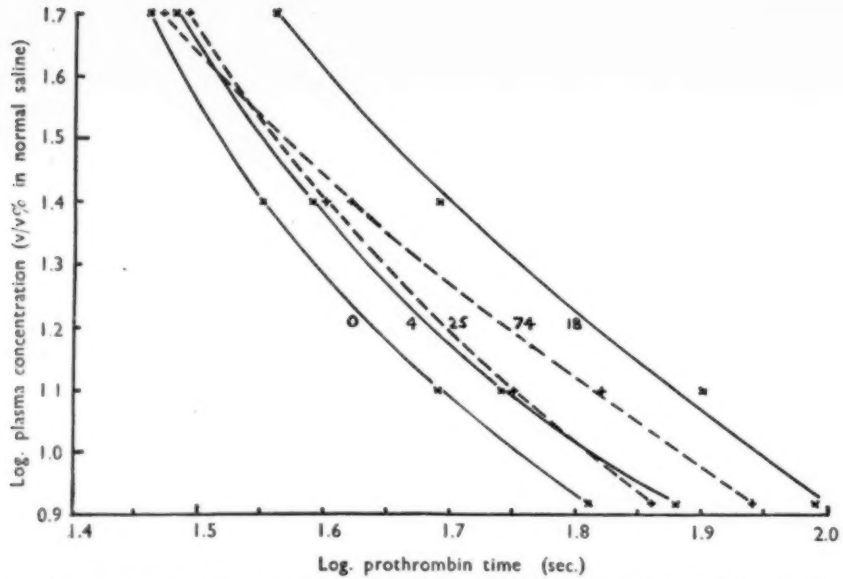


FIG. 1.—Anticoagulant effect of P.A.S. and its inhibition by streptomycin in Case 7 (A.L.). The figures against the curves give the number of days on P.A.S. before the prothrombin determination was made. On P.A.S. therapy ×————×; on combined P.A.S. and streptomycin therapy +— — — —+.

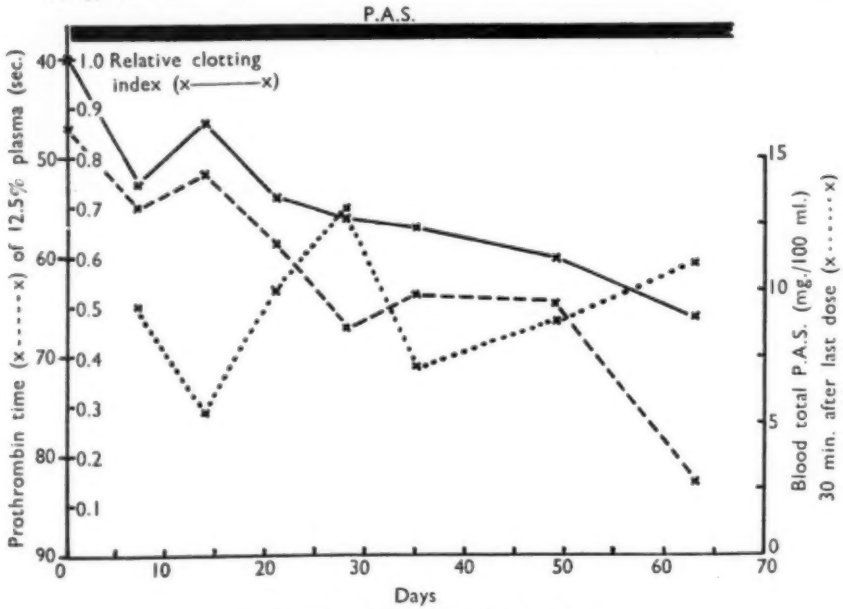


FIG. 2.—Case 4 (J.R.) treated with P.A.S.

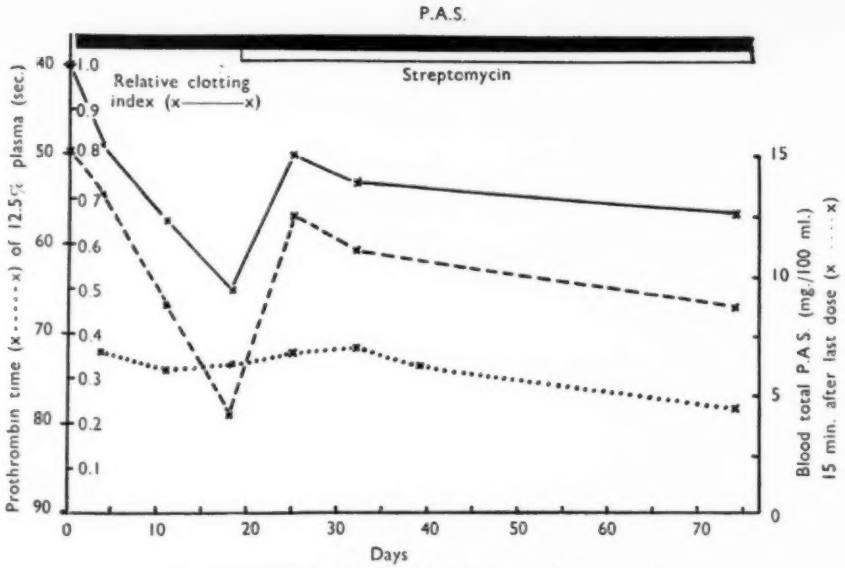

P.A.S.
Streptomycin
Relative clotting index (x———x)
Prothrombin time (x- - - - -x) of 12.5% plasma (sec.)
Blood total P.A.S. (mg./100 ml.) 15 min. after last dose (x · · · · x)
Days

FIG. 3.—Case 7 (A.L.) treated with P.A.S. and streptomycin.

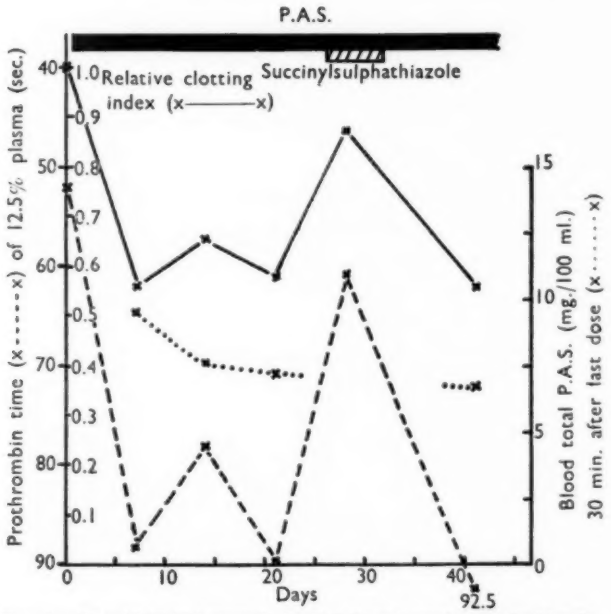

P.A.S.
Succinylsulphathiazole
Relative clotting index (x———x)
Prothrombin time (x- - - - -x) of 12.5% plasma (sec.)
Blood total P.A.S. (mg./100 ml.) 30 min. after last dose (x· · · · · x)
Days
92.5

FIG. 4.—Case 12 (R.H.) treated with P.A.S. and succinylsulphathiazole.

TABLE I

ANALYSIS OF RESULTS IN PATIENTS AND CONTROLS

Case No.	Name	Day of Observation	Prothrombin Time of 12·5% Plasma (sec.)	Relative Clotting Index	Blood Total P.A.S. (mg./100 ml.)
1	E.G.	0	42·5	1	—
			P.A.S. begun		
		6	53·5	0·66	7·3
		13	52·5	0·72	4·4
		20	59·5	0·62	6·5
		27	57	0·62	9·2
		34	56·5	0·60	6·2
		41	54	0·65	3·7
		48	55	0·67	5·9
		76	58	0·63	6·5
2	M.S.	0	41	1	—
			P.A.S. begun		
		7	54	0·69	7·4
		14	51	0·78	7·2
		21	53	0·69	8·3
		28	54	0·68	7·1
		35	48	0·81	11·0
		49	46·5	0·81	6·1
3	H.I.	0	66	1	—
			P.A.S. begun		
		7	50	1·52	7·1
		14	49·5	1·26	4·1
		21	54·5	1·29	6·4
		28	44	1·51	6·1
		35	51	1·51	4·4
		42	49·5	1·56	3·5
		56	80	0·79	7·5
		70	67·5	0·85	8·1
		84	63	1·00	6·2
5	A.G.	0	43·5	1	—
			P.A.S. begun		
		7	59·5	0·65	5·2
		Drug not tolerated; treatment discontinued			
6	B.Z.	0	47·5	1	—
			P.A.S. begun		
		7	41	1·32	9·8
Rapid deterioration; investigation not pursued, and drug withdrawn shortly afterwards					
8	G.J.	0	34	1	—
			P.A.S. begun		
		7	50	0·60	6·6
		21	61	0·42	7·8
			Streptomycin begun		
		28	49	0·66	11·4
		36	47	0·68	5·4
		64	51	0·53	4·1
9	G.B.	0	58·5	1	—
			P.A.S. begun		
		7	103	0·48	10·0
		11	67	0·74	—
Serum colloidal gold reaction, +4; thymol turbidity, 5; thymol flocculation, 2+; streptomycin begun					

TABLE I—*continued*

Case No.	Name	Day of Observation	Prothrombin Time of 12·5% Plasma (sec.)	Relative Clotting Index	Blood Total P.A.S. (mg./100 ml.)
9 *contd.*	G.B.	14	74	0·69	7·2
		21	94	0·59	6·9
		28	67	0·74	13.2
		35	69·5	0·74	6·6
		42	64	0·74	7·2
		56	59	0·87	5·5
		Serum colloidal gold reaction, +2; thymol turbidity, 4; thymol flocculation, 1+			
		75	—	—	—
		Bromsulphalein test (2 mg. dye/kg. body weight), 77·5% eliminated in 5 min.; 100% in 30 min.			
10 Control	H.T.	−9	54	1	—
		Streptomycin begun			
		0	51	1·15	—
		P.A.S. begun			
		7	56	0·95	4·1
		14	71	0·90	4·4
		28	61·5	0·86	6·3
11 Control	I.M.	0	41	1	—
		Streptomycin begun			
		5	41	1·05	—
13		0	43·5	1	—
		P.A.S. begun			
		8	59	0·72	—
		15	58·5	0·72	—
		22	62·5	0·68	—
		Succinylsulphathiazole begun			
		26	57·5	0·74	—
		29	66	0·66	—
14	E.F.	0	61	1	—
		P.A.S. begun			
		7	148	0·50	—
		Succinylsulphathiazole begun			
		11	110	0·59	—
		14	121	0·54	—
15 Normal Control	A.R.	0	52	1	—
		Succinylsulphathiazole begun			
		4	57	0·85	—
		7	58·5	0·83	—
16 Normal Control	F.R.	0	—	—	—
		Succinylsulphathiazole begun			
		4	80	0·76	—
		Succinylsulphathiazole discontinued			
		18	63·5	1	—
17 Normal Control	T.E.	0	—	—	—
		"Synkavit" begun			
		2	55	1	—
		Succinylsulphathiazole begun			
		6	67	0·78	—
		9	62	0·89	—

Plasma Prothrombin Time Estimations.—The method of Campbell, Smith, Roberts, and Link (1941) was used, with two modifications. The dried rabbit brain preparation "thromboplastin" (Evans) was used, being stored at 4° C. over phosphorus pentoxide. Stirring during the final determination of the clotting time consisted of a vertical circular movement of the platinum loop through the mixture of thromboplastin, calcium chloride, and plasma dilution, about once every three seconds, until adherent coagulum was withdrawn. More rapid stirring led to longer and irreproducible results, especially at the higher dilutions of plasma. The usual variation between duplicate determinations was from ±1 sec. for 50% plasma to ±4 sec. for 8.33% plasma. The logarithms of the prothrombin times were plotted against those of the plasma concentrations (Fig. 1). A control log.-log. curve prepared from the patient's own plasma before treatment was used to calculate the "relative clotting index" in subsequent determinations. This obviated the need for arbitrary daily "normal" controls. The "relative clotting index" (Campbell *et al.*, 1941) is the ratio of the concentration of control plasma (in the range of 12.5% to 8.33%) to the concentration of test plasma which gives the same clotting time.

Blood P.A.S. Levels.—Total P.A.S. concentrations in oxalated whole blood were determined by the method of Tárnoky and Brews (1949).

Results.—Specimen curves showing the complete results of several prothrombin determinations on Case 7 (A.L.) are given in Fig. 1. The other charts are specimen curves, showing blood P.A.S. concentrations and prothrombin levels of one patient from each treatment group. These values are recorded as Campbell's "relative clotting indices," and as the prothrombin time of 12.5% dilutions of plasma in normal saline to conform with the practice usual in this field. All other results are summarized in Table I. which also includes certain relevant laboratory findings.

Discussion

In determining the prothrombin times of 12.5% and 8.33% plasma dilutions slight variations in duplicate results make very little difference in the log.-log. curve, while in 50% plasma such variation is significant. This, and the fact that an increase in prothrombin time of 50% plasma by 5 seconds usually involves an increase of about 40 seconds at 8.33%, makes the lower end of the log.-log. curve the more accurate. This may explain the occasional crossing of curves at the 50% level, although it is possible that an apparent increase in the prothrombin content of full plasma is in fact associated with a fall as observed by the dilution method. Of the two quantities reported the 12.5% prothrombin time appears to be more sensitive, while the clotting index gives a steadier, and possibly more reliable, guide.

In four of the six cases treated with P.A.S. alone, a rapid fall in the prothrombin level was observed. This, also, was the general picture presented by cases on combined treatment. A slight rise in the prothrombin content following the initial fall in Cases 1 and 4 was accompanied by a drop in their blood P.A.S. levels. This drop may be due to decreased absorption or increased excretion of the drug during the second week, and may explain Bavin's (1949) observation in rabbits that an increase in prothrombin times after five days on P.A.S. fell to an insignificant level after 12 days. This trend, however, was clearly shown in these two cases only. Two cases (Nos. 3 and 6) showed an initial rise in the prothrombin level. This seemed to be due to long initial clotting times which have been reported in tuberculosis by Blom (1942) and by Tanner and Suter (1944). The finding was not followed up in Case 6, and there was a subsequent fall in Case 3.

Streptomycin partially reverses the antiprothrombin effect of P.A.S., the reversal being most marked at the beginning of streptomycin treatment. Later there is a smaller drop in prothrombin levels. Streptomycin administered parenterally is partly excreted in the bile (Heilman, Heilman, Hinshaw, Nichols, and Herrell, 1945 ; Robinson, 1946), and its antibacterial effect may inhibit the synthesis of dicoumarol-like substances by alimentary organisms. The temporary rise in the prothrombin level of Case 10 was probably due to clinical improvement rather than the direct effect of streptomycin, especially as this case had a low initial prothrombin level. The later slow fall in the clotting index might be due to bacterial adaptation to streptomycin or to a delayed vitamin-K deficiency also caused by the antibacterial action of streptomycin. Case 11, a second streptomycin control case, showed no significant effect of streptomycin on the prothrombin time after five days. Thus the further fall in prothrombin levels forecast by Fisher (1949) for cases on combined P.A.S.–streptomycin treatment seems to be overshadowed by the inhibitory effect of streptomycin on dicoumarol synthesis.

The administration of succinylsulphathiazole instead of streptomycin gives much less significant results. There is only a slight return of the prothrombin time towards its initial level, best shown in Case 12, and this is quickly followed by a further fall. In the three normal controls (Cases 15–17), one of whom took " synkavit " before and during the test, succinylsulphathiazole caused a fall in the clotting index. It is known that rats can be made hypoprothrombinaemic by feeding succinylsulphathiazole (Wakim, Krider, and Day, 1943 ; Day, Wakim, Krider, and O'Banion, 1943), and Poth (1945) has referred to an increased incidence of haemorrhage in patients with carcinoma of the colon when treated with this drug. A similar mechanism is reflected in our findings, which are too ill-defined from the point of view of our inquiry, and differ from the clear-cut results of analogous experiments by Jaques and Lepp (1947).

Oral P.A.S. therapy thus has a definite antiprothrombin effect, and our results serve to confirm the findings of other observers. Also P.A.S. seems similar to salicylates in its anticoagulant effect. This latter effect may be explained by Jaques and Lepp's theory rather than Link's. The findings of Barclay (1949), however, still await explanation, especially as they correspond to the results of Link *et al.* (1943) working with salicylate administered intravenously. These suggest a direct effect due to P.A.S. itself. If salicylates act only through their relation to dicoumarol-like substances, a view Jaques and Lepp do not hold, then, short of a more direct synthesis, a large-scale excretion of parenteral P.A.S. into the alimentary tract would be necessary.

The clinical significance of this anticoagulant effect has been dealt with by Madigan *et al.* (1950) and Lynch (1950). We may add, however, that P.A.S. has a more marked effect in patients who are in poor condition and especially in cases with liver damage. Such patients generally have longer initial clotting times. The changes due to P.A.S. treatment are reduced by the present practice of giving P.A.S. and streptomycin together. The use of vitamin K is of course no less justified than in treatment with P.A.S. alone.

Summary

The effect of oral sodium *p*-aminosalicylate on the prothrombin level was examined in 13 tuberculosis patients.

Six cases received P.A.S. alone, four were on a combined course of P.A.S. and streptomycin, and three were given succinylsulphathiazole at a point during their course of P.A.S.

P.A.S. caused a definite lowering of prothrombin levels as determined on diluted plasma. This effect was partly reversed by streptomycin. Succinylsulphathiazole gave inconclusive results.

These findings can be explained by the theory of Jaques and Lepp (1947) rather than by that of Link *et al.* (1943); P.A.S., like salicylates, may be changed to a dicoumarol-like compound by intestinal bacterial action.

We should like to thank Dr. E. N. Allott, Director of the Group Laboratory, Lewisham Hospital, S.E.13, for valuable advice and criticism; Dr. J. H. F. Wood and Miss V. A. L. Brews for help in the experimental work; and the medical staff of St. Andrew's Hospital, E.3, who made their cases available to us and acted as controls for these observations.

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TECHNICAL METHODS

AGGLUTINATION OF *H. PERTUSSIS* USING CONCAVITY SLIDES

BY

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The agglutination reaction is the easiest to perform and is probably the most widely used of the serological tests for the detection of *H. pertussis* antibodies. Two methods have been used, a slide test and a tube test. A comparison of these techniques has been made by a number of workers (Evans and Maitland, 1939; Mishulow, 1942; Powell and Jamieson, 1942; Powell, 1944; and DeGara and Mayer, 1947). A summation of these results suggests that, for agglutination, the slide test is more useful as a diagnostic or screening test, and tube agglutination is more suitable for quantitative work.

The Laboratory of Hygiene is currently conducting studies on the immunization of children against diphtheria, tetanus, and whooping-cough. The volume of the individual serums is frequently limited, so that tests requiring small amounts of sera are of definite advantage. For this reason the practicability of a slide test for the determination of *H. pertussis* agglutinins was considered. The uncovered slide technique was found unsuitable for quantitative work because the test materials dried rapidly and end-results were difficult to interpret. It was therefore decided to investigate the usefulness of concavity slides, covering the depressions with glass cover slips to prevent evaporation. The results of these tests are described in this paper.

Procedure

Materials.—The following were used:

Concavity Slides.—Slides* measuring 2 × 3 in., with 10 polished concavities, 15 mm. in diameter and 1.5 mm. deep, were used.

Cover Slips.—Circular microscope cover-slips were used, having the same diameter or a slightly smaller one than that of the depressions. Cover slips with larger diameters were found unsuitable as they were easily displaced during the rotation, resulting in the loss of test materials.

Rotating Machine.—Because of its convenience, a Fisher-Kline rotator timed to run for four minutes at 280 revolutions per minute was used. Other rotators giving a similar rotary motion in a horizontal plane might prove equally satisfactory.

Pipettes.—Pasteur pipettes with tips carefully prepared so as to deliver drops of constant size were used. The number of drops per test varied, depending on the diameter of the tip.

* Obtained from Clay Adams Co.

Antigen.—A commercial antigen* diluted in normal saline to contain 1×10^{10} organisms per ml. was used. It consists of killed Phase I *H. pertussis,* preserved with ethyl mercuric thiosalicylate at a dilution of 1 : 10,000 and coloured with methylene blue. Other suspensions prepared in this laboratory were tried, but did not prove as satisfactory.

Method.—The method for the two tests is as follows :

Qualitative Test.—The serum to be tested is drawn up in the Pasteur pipette and two drops placed in a cavity. The pipette is then rinsed several times in saline, and the tip is blotted with some absorbent material such as filter paper to ensure more complete drying. The same pipette should be used for a complete test to minimize the error. An equal quantity of vaccine is added and the cover slip placed over the cavity. The cover slip is held in place by the liquid. If the proper amount of material is used, an air space appears under the cover slip, and this aids in mixing the antigen and serum during the rotating. For control purposes, to ensure against auto-agglutination, a mixture of the agglutinable suspension and saline is included on each slide. As a further control, the vaccine is checked with known negative serum. The latter was performed on each day of test but not on each slide.

With a pipette approximately 0.050 to 0.075 in. in diameter, we have found that four drops almost fill the cavity. If too much fluid is added the cover slip will not fit.

Quantitative Test.—Twofold dilutions of the sera to be tested are made directly on the slide. Two drops of saline are placed in each cavity. Two drops of serum are added to the first cavity and the liquid is mixed by raising and expelling it three times with the Pasteur pipette. Two drops of the mixture are then transferred to the next cavity and so on until the required number of dilutions are made. The antigen is added as previously described, and the cover slips are put in place. The slide is then put on the rotating machine.

In both the qualitative and quantitative tests the slides are rotated for four minutes and then held at room temperaure for two hours before reading.

Tests conducted over temperature ranges from 5° C. to 54° C. showed that higher titres were obtained at 30° C. and 37° C. Further, it was found that the combination of a four-minute rotation period and incubation at 37° C. yielded the highest titres in these experiments.

The results are most easily read if the slides are held up to the light and read against a dark background. Fig. 1 shows a typical agglutination reaction.

Discussion

The test described has been used successfully for the determination of *H. pertussis* agglutinins in sera from infants and test animals. The technique described was used by Greenberg and Fleming (1950) for the determination of pertussis agglutinins in a series of immunization studies with children. None of the sera was found to have pertussis agglutinins before immunization. Wide variations in titre from 0 to 1 : 4096 were found in the post-immunization sera.

The test has proved practical and requires only small quantities of material. The reactions were easy to read and yielded reproducible results. The results compared favourably with those in the tube test (Table I), and were obtained in a much shorter time. The procedure used for the tube test was essentially the same as Miller and Silverberg's method (1939).

* Eli Lilly & Co., Indianapolis.

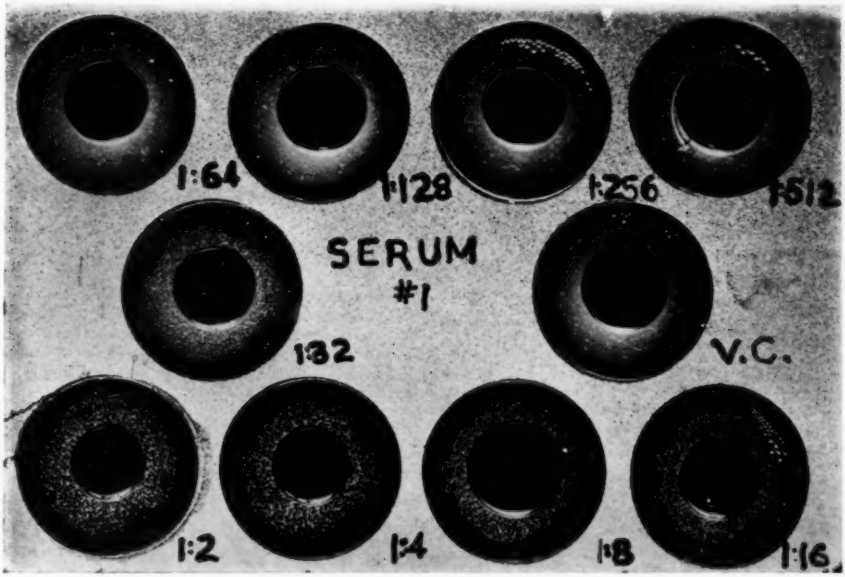

FIG. 1.—Agglutination on a concavity slide. The agglutination end-point is 1 : 32 dilution. V.C. is the vaccine (antigen) control.

TABLE I

COMPARISON OF RESULTS IN SLIDE AND TUBE TESTS FOR DETERMINATION OF PERTUSSIS AGGLUTININS

Serum Number	Tube Test Titre Reading after 19 Hours' Refrigeration	Concavity Slide Titre Reading after 2 Hours at Room Temperature
25	1 : 16	1 : 16
29	1 : 16	1 : 16
30	1 : 16	1 : 16
63	1 : 128	1 : 128
90	1 : 32	1 : 32
339	1 : 64	1 : 64
343	1 : 128	1 : 256
346	1 : 16	1 : 32
B.T.	1 : 16,384	1 : 16,384

It was also found that rotating accelerated the rate of agglutination, and that by rotating for four minutes the maximum titre was reached 45 minutes earlier than control slides not rotated. The rotation made the reaction stronger and easier to read, but did not change the final agglutination titre.

The described technique has been used only for *H. pertussis* agglutination, but with modifications it could be applied to other agglutination tests performed as a routine in laboratories.

Summary

H. pertussis agglutinations, both qualitative and quantitative, were successfully completed using concavity slides with covered depressions. The results compared favourably with those in the tube test and were obtained in a much shorter time.

The highest agglutination titres were obtained by rotating the slides for four minutes and incubating at 37° C. Final readings could be taken in approximately one hour.

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A SIMPLE TITRATION METHOD FOR DETERMINING THE SPECIFIC GRAVITY ON ONE DROP OF URINE

BY

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Samples of urine which have been collected by ureteric catheterization are necessarily small in volume, often only a few drops. In order to determine the efficiency of kidney function it may be necessary to measure the specific gravity of these small samples, but it is impossible to use the hydrometer method, which requires a minimum of about 50 ml. of urine. Methods involving the suspension of drops of urine in solvents, which are immiscible with water, are a possible alternative. Dukes (1939) has described a method based on these principles, but it has some disadvantages. A drop of urine is added to a mixture of carbon tetrachloride and xylol, and the composition of the mixture is adjusted by adding one of the constituents until the drop of urine floats in an equilibrium position. The specific gravity of the mixture is then measured with a urinometer. This requires the use of large volumes of reference liquid, 50 ml., for each test, and errors may be introduced due to the fact that the slight solubility of water in the 50 ml. of liquid may appreciably alter the composition of the drop of urine. The method of Monroe and Hopper (1946) makes use of a series of standard mixtures of xylol and bromobenzene of known specific gravity. Drops of urine are added to these mixtures in turn until the equilibrium mixture is found. This method is open to the same possibility of error, due to the relatively large volume of reference liquid used, as is the first method. Several drops of urine are required for the test and may not always be available. There is also the disadvantage that it is necessary to keep a large number of standard mixtures.

Experimental

The principle of the proposed method is as follows. One drop of urine is placed in a standard, small volume of an immiscible, inert liquid which has a specific gravity slightly lower than the minimum value for urine. The drop will sink to the bottom of the liquid. A denser liquid is added from a microburette until a mixture is obtained in which the drop of urine floats in equilibrium. The volume of liquid added is used to obtain the specific gravity from a graph.

The reference liquids should have the following properties. They should be readily miscible with each other but have a low solubility for water. No chemical reaction should take place between the two liquids or the urine. The component liquids should be stable, chemically pure substances with constant specific gravities

in order that the specific gravity of any mixture of known composition can be calculated. They must have suitable specific gravities to cover the extreme limits of that of urine, 1.000 to 1.050. The lighter of the two liquids must have a specific gravity which is lower than that of any urine which may be submitted for testing so that the drop of urine will sink in it. There is no point in using a liquid with a specific gravity much below 1.000, as the method would lose sensitivity. A value of about 0.995 would be suitable. The liquids used in the methods referred to above would not be satisfactory. Xylol is not a pure chemical substance but a mixture of three xylenes ; its composition, and hence its specific gravity, may vary in different batches, and this would interfere with the calibration. Moreover, xylol has too low a specific gravity, about 0.870. A mixture of equal volumes of benzene and monochlorobenzene has a specific gravity of 0.992, and this has been found to be satisfactory as the first reference liquid. These components are both readily obtainable pure and are perfectly stable. The other, denser reference liquid should not have too high a specific gravity. It can be seen in Fig. 1 that the liquids used in other methods, carbon tetrachloride and bromobenzene, have unnecessarily high specific gravities, 1.600 and 1.499 respectively. In order to get maximum sensitivity it is essential that

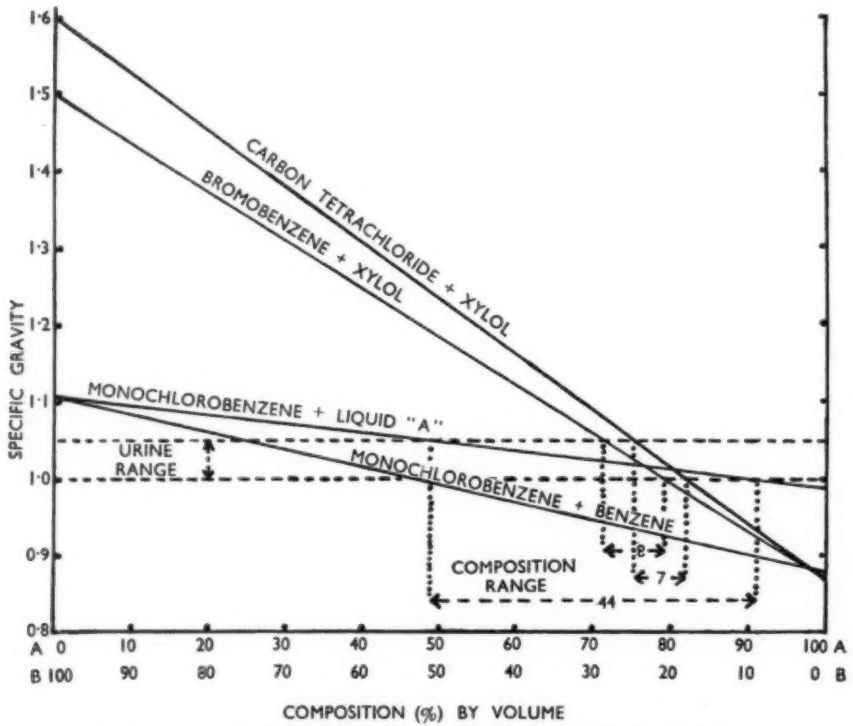

FIG. 1.—Graph of specific gravities of liquid mixtures of different composition.

there should be a large change in the composition of the mixtures from a specific gravity of 1.000 to 1.050. Monochlorobenzene, which has a specific gravity of 1.107, has been found to be most suitable for this method.

Method and Materials

Materials.—The following materials are required.

Monochlorobenzene.—It should be pure, redistilled, and with a boiling point of 132° C. and specific gravity of 1.107.

Benzene.—Benzene should be pure, redistilled, with a boiling point of 80.1° C. and a specific gravity of 0.879.

Reference Liquid "A."—This should be of a specific gravity of 0.992, prepared by mixing 50 ml. benzene with 50 ml. monochlorobenzene.

Calibration.—The composition of the mixtures obtained by adding different amounts of monochlorobenzene to 1 ml. of reference liquid "A" was calculated. The specific gravities of these mixtures were obtained from a graph. The results are given in Table I. The calibration curve was prepared by plotting a graph of the specific gravities of the mixtures against the volume of monochlorobenzene *added* (columns 6 and 2, Table I).

TABLE I

COMPOSITION AND SPECIFIC GRAVITIES OF MIXTURES OBTAINED BY ADDING DIFFERENT AMOUNTS OF MONOCHLOROBENZENE TO ONE MILLILITRE LIQUID "A"

Volume of Monochlorobenzene in One Millilitre of Liquid "A" (ml.)	Volume of Monochlorobenzene Added in Titration (ml.)	Total Volume of Monochlorobenzene (ml.)	Total Volume of Liquid (ml.)	Percentage of Monochlorobenzene in Mixture	Specific Gravity of Mixture
0·5	0·05	0·55	1·05	52·4	0·997
0·5	0·10	0·60	1·10	54·5	1·002
0·5	0·15	0·65	1·15	56·5	1·007
0·5	0·20	0·70	1·20	58·3	1·011
0·5	0·25	0·75	1·25	60·0	1·015
0·5	0·30	0·80	1·30	61·5	1·019
0·5	0·40	0·90	1·40	64·3	1·025
0·5	0·50	1·00	1·50	66·7	1·031
0·5	0·60	1·10	1·60	68·9	1·036
0·5	0·70	1·20	1·70	70·6	1·040
0·5	0·80	1·30	1·80	72·3	1·044
0·5	0·90	1·40	1·90	73·7	1·047
0·5	1·00	1·50	2·00	75·0	1·050

Method.—Into a 3 × ½-in. test-tube 1 ml. of the reference liquid "A" is accurately measured, allowing sufficient time for the pipette to drain properly. One small drop of the urine is added to the liquid by means of a capillary pipette. The urine falls to the bottom of the tube. Monochlorobenzene is added drop by drop from a 2-ml. microburette graduated in 0.01-ml. divisions. After the addition of each drop, the liquid in the tube is gently swirled in order to mix the liquids and also to stir up the drop of urine from the bottom of the tube. The behaviour of the drop of urine is observed when the tube is stationary. If it falls again to the bottom of the tube, more monochlorobenzene is added and the swirling and observation repeated. The end-point is reached when the

drop of urine no longer falls, but remains suspended in the liquid or rises very slowly when the tube is at rest. The volume of monochlorobenzene added is noted, and the specific gravity of the urine is read from the calibration curve.

The swirling movement of the liquid should not be so vigorous that the drop of urine is broken up into very small droplets, as this makes it more difficult to see when the end-point is reached. With a little practice it is possible to determine the end-point within one small drop of monochlorobenzene, which is equivalent to approximately 0.001 specific gravity.

Errors due to the urine-water dissolving in the organic liquids are negligible because only small volumes, 1 to 2 ml., of these are used. There is also the obvious advantage of economy in materials. The used mixtures may be collected in a residues bottle and subsequently recovered by fractional distillation. The boiling points of the two liquids are sufficiently different to permit of their easy purification.

The reference liquids are stable indefinitely.

Results.—Random samples of urine were collected from ward patients. The specific gravity of each specimen was determined by the titration method using one drop. Subsequently, the specific gravity was measured with a urinometer in the bulk of the specimen. Comparison of the results in Table II shows satisfactory agreement between the two methods. The urinometer readings do not represent absolutely correct values, and therefore differences in the results obtained by the two methods must not be ascribed entirely to error in the micro method. It will be seen below that these results may be on opposite sides of the true value.

TABLE II

COMPARISON OF SPECIFIC GRAVITY READINGS OF URINE SAMPLES BY MICRO-TITRATION AND BY URINOMETER

Specimen No.	Titration (ml. of Monochlorobenzene)	Specific Gravity Reading from Calibration Curve	Specific Gravity Reading by Urinometer
1	0·24	1·014	1·014
2	0·31	1·020	1·019
3	0·32	1·020	1·017
4	0·44	1·027	1·023
5	0·28	1·017	1·018
6	0·26	1·016	1·016
7	0·31	1·020	1·018
8	0·89	1·047	1·043
9	0·20	1·011	1·009
10	0·27	1·017	1·016
11	0·30	1·019	1·017
12	0·17	1·009	1·010
13	0·32	1·020	1·016
14	0·19	1·010	1·012
15	0·23	1·013	1·016

In order to test the accuracy of the method over the whole range of specific gravities likely to be encountered in urines, salt solutions were used. The specific gravities of a series of solutions ranging in concentration from 1 to 7% of sodium chloride in distilled water were measured by three methods for comparison ; first, the micro-titration method as described, secondly by urinometer, and thirdly by a gravimetric method. For the last method, a sample of the solution was accurately measured in a 25-ml. pipette, transferred to a tared weighing bottle, and weighed on an analytical balance to the nearest

0.0005 g. Using the same pipette, cleaned and dried, the same volume of distilled water was weighed in a similar manner. The specific gravity of the salt solution was calculated by dividing the weight of the sample by the weight of the same volume of water. In this way, possible errors in the calibration of the pipette are compensated. This method is the most accurate of the three used and can be taken as a reliable basis for comparison.

The results in Table III show that the micro-titration method is reliable within ±0.002 unit of specific gravity over the whole range which is likely to be found for urine, from 1.006 to 1.047.

Very occasionally, urines with very high specific gravity have been received from patients who have had "uroselectan." The actual values of specific gravity of these urines have no clinical significance, and it has not been considered advisable to alter the range of the method to include these.

All values of specific gravity in this paper are for a room temperature of 20° C.

TABLE III

COMPARISON OF RESULTS FOR THE DETERMINATION OF THE SPECIFIC GRAVITIES OF SALT SOLUTIONS BY THREE METHODS

Concentration of Sodium Chloride (%)	Micro-titration Method		Urinometer Method (Specific Gravity)	Gravimetric Method (Specific Gravity)
	Titration (ml.)	Specific Gravity		
1·0	0·15	1·007	1·006	1·0060
2·0	0·20	1·011	1·015	1·0130
3·0	0·29	1·018	1·020	1·0203
4·0	0·46	1·028	1·028	1·0268
5·0	0·53	1·032	1·033	1·0331
6·0	0·79	1·044	1·043	1·0433
7·0	0·86	1·046	1·045	1·0467

Summary

A simple titration method for the determination of the specific gravity of urine when only one drop is available is described.

One drop of urine is added to a small volume of a mixture of benzene and monochlorobenzene, in which it sinks. The composition of the mixture is altered by titration with monochlorobenzene until the urine floats in equilibrium. The specific gravity of the urine is read from a calibration curve.

Results are given to show that the method is reliable within 0.002 unit of specific gravity over the range 1.006 to 1.047.

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REVIEWS

Introduction to Neuropathology. By Samuel P. Hicks and Shields Warren. 1950. London, New York, and Toronto: McGraw-Hill Publishing Co., Ltd. 1st ed., illustrated. Pp. 494. Price 85s. ($10.00).

This account of neuropathology differs from most in that the authors have endeavoured to bring the subject more into line with that of the physiology and pathology of the body as a whole. It is written by men of wide experience, so that while it sets out to be only an elementary treatise, up-to-date views are freely given and criticized and the whole is written with a strong functional and practical bias; thus the pathologist who is called upon from time to time to report upon a cerebral tumour will find much help within these pages.

The volume is lavishly illustrated and the captions provide interesting reading. Each chapter includes a useful bibliography. The complicated method of numbering the illustrations beginning each chapter independently is, however, confusing and offers no apparent advantage.

W. McMENEMY.

Atlas of Tumor Pathology: Sect. II, Fasc. 6. Tumors of the Peripheral Nervous System. By Arthur Purdy Stout. 1949. Washington, D.C.: American Registry of Pathology. Price 60 cents.

This fascicle, the first of the series to be issued, comprises 57 loose-leaf pages of text and illustrations. The latter, 56 photographs in black and white, are excellently produced and include some of Dr. Margaret Murray's beautiful tissue-cultures. As a recognized authority in this branch of oncology, Dr. Stout's contribution is assured of a wide welcome.

The preparation of an atlas of this kind is a noble project indeed, envisaging the issue of some 39 fascicles in 11 sections covering the subject in a regional manner, and prepared by a large team of experts. The fascicles are to be issued in no particular order, but will appear when they are ready. As a government publication it is being made available at cost price, and can be obtained by application to the American Registry of Pathology, Armed Forces Institute of Pathology, Washington, D.C. It is much to be hoped that laboratories in this country will find no obstacle placed in the way to obtaining what promises to be a most valuable work of reference.

DOROTHY S. RUSSELL.

Practical Section Cutting and Staining. By E. C. Clayden. 1951. London: J. and A. Churchill, Ltd. 2nd ed. with 23 illustrations. Pp. 135. Price 10s.

The issue of a second edition in less than three years from its first publication is a tribute to the good reception enjoyed by this manual. The text has been revised without essential alteration of its general form and lay-out. New methods added include the rapid preparation of bone sections, Nile-blue stain for fats, Busch's modification of Marchi's method, and toluidin blue for Nissl granules. The revision has entailed the substitution of a few more up-to-date methods for those originally given; for example, Gordon and Sweet's method for reticulum fibres in place of Laidlaw's. In parenthesis, "reticulin" is generally preferred to "reticulum" in this context. The author states (p. 85) that "the tissue" (C.N.S.) "has a great tendency to wash up during the process of staining." This alleged disadvantage, couched in the same terms in the first edition and therefore presumably not a printer's error, needs redemption from obscurity! In general, however, the text is clear and is the handiest small guide for the histological technician in his early years.

DOROTHY S. RUSSELL.

McClung's Handbook of Microscopical Technique. Edited by Ruth McClung Jones. 1951. London: Cassel & Co., Ltd. 3rd ed. 157 plates. Pp. 790. Price 90s.

Pathologists are, unfortunately, becoming only too conscious that the reference books which they would like to own are issued at a price that is prohibitive. *McClung's Handbook* is of established value, and perhaps institutional libraries at least ought to afford a copy of the new edition, the first published after the original editor's death.

It contains much that the histologist and haematologist in particular cannot find conveniently in other textbooks; there is a particularly full chapter on polarized light and electron microscopy, and a useful but brief treatise on phase contrast, in which the kind of lamp needed is not adequately stressed. Radio-autographic technique and dental histology are fully discussed. Quotations from non-American literature are few; the paper curtain perhaps excludes them. The volume comprises, however, a very fair balance between a record of tried methods and an account of new research weapons.

In a book published at this price it is unfortunate that certain pages should be out of sequence.

R. J. V. PULVERTAFT.

La Talassemia. By G. Astaldi, P. Tolentino, and C. Sacchetti. 1951 Pavia: Biblioteca "Haematologica." No. XII. With 69 illustrations. Pp. 229. Price Lira 2,500.

"Thalassaemia," an expressive if philologically unfortunate neologism, is becoming accepted in haematological literature as a term embodying both the fatal haemolytic anaemia of Cooley and the less severe hereditary Mediterranean target cell anaemias described in recent years in Italy and the United States. There seems little doubt that both disorders are expressions of the same underlying gene mutation and that a fatal outcome occurs in homozygous individuals, whereas in heterozygotes the condition may range from clinical silence to a severe anaemia with skeletal changes indistinguishable from those found in the true "Cooley" type. The disease is widespread along the Mediterranean littoral and in people of Mediterranean stock in other countries. Changes thought to be characteristic of it have been discovered in skeletons dating from palaeolithic times. Thalassaemia is thus of interest to the haematologist, the geneticist, and the historian, and in their monograph Astaldi, Tolentino, and Sacchetti have assembled most of the known facts about it. This excellent book contains a full review of the literature—the great bulk Italian—good accounts of the clinical and haematological features of the disease, and a résumé of much of the authors' original work designed to elucidate its precise pathogenesis. Their conclusion, drawn partly from accurate studies of the bone marrow and of bile pigment metabolism, partly from tissue culture experiments, is that the primary lesion of thalassaemia lies in an increased destructibility of the abnormal erythrocytes, with a consequent shortening of their life span and a compensatory hyperplasia of the marrow erythroblasts. The weakness of the argument lies in the lack of direct evidence for a shortened survival of the erythrocytes. Such evidence has recently been produced by DeGowin's group by means of cross-transfusion experiments, and it has also been suggested that this does not fully account for the anaemia which is more likely secondary to a partial haemopoietic arrest of the marrow. These additional findings amplify the value of Astaldi, Tolentino, and Sacchetti's results.

The book is well produced on good paper. The illustrations, especially the photomicrographs, are of the high standard customary in Italian publications. There is a useful if somewhat unidiomatic summary in English and a very extensive list of references. This is a work which should be available to everyone interested in the study of the haemolytic anaemias.

F. W. GUNZ.

J. clin. Path. (1951), **4**, 498.

ABSTRACTS

This section of the JOURNAL *is published in collaboration with the two abstracting journals, Abstracts of World Medicine, and Abstracts of World Surgery, Obstetrics and Gynaecology, published by the British Medical Association. In this* JOURNAL *some of the more important articles on subjects of interest to clinical pathologists are selected for abstract, and these are classified into four sections: bacteriology; biochemistry; haematology; and morbid anatomy and histology.*

BACTERIOLOGY

Use of Penicillin O in Patients Hypersensitive to Penicillin G. VOLINI, I. F., SHALES, W. H., and FELSENFELD, O. (1950). *J. Amer. med. Ass.*, **143**, 794.

Several workers have reported that persons hypersensitive to penicillin G may show no reaction to some of the biosynthetic penicillins. One of these is allylmercaptomethyl penicillin, or penicillin O. The minimum inhibitory concentrations of penicillin O for Gram-positive cocci are the same as, or double, those of penicillin G. Furthermore, penicillin O was found to be less toxic and less irritating than penicillin G when injected subcutaneously into mice. The absorption and excretion curves of these two penicillins are essentially the same.

Penicillin O was administered to 57 patients who had clinical symptoms of hypersensitivity to penicillin G within a preceding period of not more than 200 days. Twenty of them had upper respiratory infections, and were given inhalations of penicillin-O dust, 100,000 units every 4 hours for 7 days. All recovered without complications and showed no allergic phenomena. In 23 cases of tonsillitis and pharyngitis due to β-haemolytic streptococci the patient received 10 troches containing 3,000 units of penicillin O daily for 3 days. The results were excellent in 15, good in 5, and poor in 3 cases. Fourteen patients received courses of injections of penicillin O, following which penicillin G was given and was well tolerated. It appears that penicillin O had a desensitizing effect. Seven of the cases are reported in detail and a further 7 in tabular form. In every case penicillin O was as effective as penicillin G.

A. W. H. Foxell.

Chloramphenicol in the Treatment of *Hemophilus influenzae* Meningitis. PRATHER, G. W., and SMITH, M. H. D. (1950). *J. Amer. med. Ass.*, **143**, 1405.

From the Charity Hospital, New Orleans, the authors report the treatment with chloramphenicol of 15 consecutive, unselected cases of *Haemophilus influenzae* meningitis. Of these 15 patients 8 were infants under one year. In every case *H. influenzae* type B organisms were recovered in culture from the spinal fluid, but fluid from a second puncture performed within an average interval of 21 hours was sterile. All the patients recovered, no toxic effects attributable to the drug being observed. On the basis of this experience the authors recommended immediate doses between 50 and 100 mg. per kg. body weight by mouth or stomach-tube, followed by 250 mg. 8-hourly for 5 or more days irrespective of body weight. They consider this method of treatment to be more satisfactory than any other known to them.

Joseph Ellison.

Synthetic and Fermentation Type Chloramphenicol (Chloromycetin) in Typhoid Fever: Prevention of Relapses by Adequate Treatment. SMADEL, J. E., BAILEY, C. A., and LEWTHWAITE, R. (1950). *Ann. intern. Med.*, **33**, 1.

A continuation of the studies on the use of chloramphenicol ("chloromycetin") in

the treatment of patients with typhoid fever indicates that the synthetic form of the drug is as efficacious as the natural antibiotic obtained by the fermentation process from *Streptomyces venezuelae*. The same total amounts of either type drug are equally effective when given in divided doses at 2- to 6-hour intervals or in larger doses once or twice daily.

There was a definite relationship between the duration of chloramphenicol treatment and the occurrence of relapses in typhoid fever. Slightly more than half of the patients who were treated for 8 days or less had a recrudescence of the disease which began about 10 days after treatment was stopped. No relapses occurred in the groups of patients treated for longer periods of time. The present data suggest that a 14-day period of treatment is sufficient to prevent relapses. In spite of the dramatic therapeutic effectiveness in patients with typhoid fever, serious complications such as intestinal haemorrhage and perforation may be expected in treated patients, since the stage is generally set for such developments before therapy is instituted and time is required for the healing of the typhoidal lesion of the intestine. In the present group of 23 patients, 2 had haemorrhage sufficiently severe to produce shock. Two other patients suffered intestinal perforation; the course in one of these was further complicated by severe haemorrhage and the disease terminated fatally. Neither of the patients with perforation was given surgical treatment; chloramphenicol therapy controlled or suppressed the usual signs of generalized peritonitis.

On the basis of the present observations, it would appear that the adequate treatment of typhoid fever in the adult consists of an initial oral dose of 3·0 to 4·0 g. of chloramphenicol, followed by 1·5 g. oral doses given every 12 hours during the febrile period and by single daily 1·5 g. doses for 7 days; thereafter the dose may be reduced to a single 1·0 g. dose and continued until the 14th day of antibiotic therapy, after which the drug may be discontinued. Particular attention should be given to the recognition of intestinal perforation in treated patients, since the classical signs of this development with the ensuing generalized peritonitis may be partially masked by the antibacterial effect of chloramphenicol.—[Authors' summary.]

Treatment of Whooping-cough with Antibiotics (Streptomycin, Aureomycin, and Chloramphenicol). FREYRE, A. VIDAL (1950). *Arch. argent. Pediat.*, **34**, 284.

Streptomycin was given in the treatment of 20 children with whooping-cough in a dose of 0·5 g. intramuscularly, repeated every 12 hours, to a total of from 3 to 5 g. Results are classified as very good in 4 cases, good in 9, fair in 5, and unsatisfactory in 2. In one of these failures rapid cure was achieved with aureomycin. Chloramphenicol was given to 5 infants and children varying in age from 4 months to 3½ years, and to one adult; aureomycin was given to 9 infants and children aged from 2 months to 7 years of age. The dosage by mouth of chloramphenicol was identical with that of aureomycin: 50 mg. per kg. body weight every 6 hours for the first 2 or 3 days and every 8 hours later. With aureomycin results were excellent in 6, good in 1, and only mediocre in the other 2. With chloramphenicol the results were spectacular, and after the third or fourth dose there were practically no further symptoms. *G. M. Findlay.*

General Remarks on 300 Cases of Typhoid Fever Treated with Chloramphenicol and Associated Medication. BENHAMOU, E., DESTAING, F., and SORREL, A. (1950). *Sem. Hˆp. Paris*, **26**, 4107.

The results of treating a series of 200 cases of typhoid and paratyphoid fevers in North Africa with chloramphenicol are compared with those obtained in a previous series of 100 cases. The mortality has decreased progressively; of the first 100 patients 86 were cured, of the second 100 patients 94, and of the last 100 cases 98. These increasingly good results are attributed to observance of the following principles. Antibiotic treatment should be begun as soon as the

clinical symptoms suggest typhoid fever and before waiting for a bacteriological diagnosis. As a general rule no attempt should be made to give a large loading dose of chloramphenicol: 3 g. in the first 2 hours is quite enough for an adult and is approximately equivalent to 50 mg. per kg. body weight. Aureomycin should be given at the same time as chloramphenicol, as it reduces greatly the risk of collapse. A large loading dose of chloramphenicol may, however, be given without any danger provided the patient's illness has lasted only a few days. After the first week a loading dose should never be given. A dose morning and evening is usually sufficient to produce prompt improvement. For adults 1·5 g. morning and evening, and for children 0·25 g. at the same time is sufficient dosage. With more effective treatment, relapses have become less common, provided that chloramphenicol has been given in decreasing doses for 14 days after the temperature has reached normal. In the first 100 cases there were 14 relapses: in the second 100 cases 12, and in the third 5. Relapses may occur very late in convalescence, and in 2 cases in the present series developed, with positive blood culture, 60 and 72 days respectively after the temperature had fallen to normal.

It is concluded that ordinary typhoid fever can invariably be cured by chloramphenicol, the temperature returning to normal in 3 days. There are, however, three complications which do not respond to chloramphenicol alone. These are intestinal complications characterized by meteorism, intestinal haemorrhage, and perforation; cardiac failure, with tachycardia and vascular collapse; and encephalitis, with delirium, stupor, and myoclonic contractions. Some evidence is brought forward to show that these symptoms are caused by strains of typhoid bacilli with a low sensitivity to chloramphenicol. The best results are obtained by giving a mixture of chloramphenicol, aureomycin, and sometimes also streptomycin. [It should be noted that chloramphenicol is now known in France as " tyfomycine."] *G. M. Findlay.*

Chloramphenicol (Chloromycetin) Therapy in Shigella Enteritis. ROSS, S., BURKE, F. G., RICE, E. C., WASHINGTON, J. A., and STEVENS, S. (1950). *J. Amer. med. Ass.*, **143**, 1459.

Chloramphenicol has been used in the treatment of 35 children, aged between 3 months and 7 years, suffering from bacterial dysentery (due in 33 cases to *Shigella sonnei* and in 2 to *Sh. flexneri*). Tests of sensitivity to the drug were made on the isolated organisms, which all proved sensitive to 5 μg. per ml. or less. Each case was confirmed bacteriologically before treatment was started, and daily stool cultures were made throughout the stay in hospital. In general, the dosage employed was 250 mg. 4-hourly for about 8 days. In all but 2 of the 35 cases stool cultures became negative within 12 to 36 hours of starting treatment. Of the other 2 the stools of one became negative in 48 hours and of the other in 6 days, with a relapse 8 days after discharge. He was treated again with 500 mg. of chloramphenicol 4-hourly for 10 days and recovered completely. Clinically, the disease subsided *pari passu* with the bacteriological findings, much improvement usually being seen within the first 24 hours, though extensive fluid therapy was needed in a few cases, mostly in infants. Diarrhoea subsided within 3 days and all the patients were discharged cured after 8 consecutive negative bacteriological reports on the stools. Side-actions seem to have been chiefly due to the bitter taste of the drug, many of the patients being too young to take the capsule intact. Anorexia and vomiting were observed in a few cases, but were only transitory. In 4 female patients vulvovaginitis was observed about 5 days after starting treatment, but it cleared up when the drug was stopped. Comparison is made with the results of treatment with other drugs. It was found that sulphadiazine, streptomycin, polymyxin B, and chloramphenicol all produced negative bacteriological results within 2 days, the average figures being 2·0, 1·6, 1·9, and 0·76 days respectively, and the differences being statistically insignificant. There are, there-

fore, four drugs now available which may be expected to clear up bacterial dysentery quickly and completely.

Reginald St. A. Heathcote.

Chloramphenicol in the Treatment of the Acute Manifestations of Brucellosis. KNIGHT, V., RUIZ-SANCHEZ, F., and MCDERMOTT, W. (1950). *Amer. J. med. Sci.*, **219**, 627.

The authors studied 13 patients suffering from brucellosis who were treated with chloramphenicol. In general, the dosage was 50 to 100 mg. per kg. daily during the febrile period, followed by 25 mg. per kg. daily up to a total period of 6 to 10 days. In 5 patients there was a rapid subsidence of fever and they remained well. There was an early improvement in symptoms in 2 patients, both with bacteriaemia and both severely ill, but fever continued after chloramphenicol was stopped. In one of these 2 cases a second course of treatment instituted immediately resulted in rapid and apparently permanent improvement. In the other there was a long period of low-grade fever; later there was a prompt and beneficial response to a second course of the drug. Relapses occurred in 6 cases within 1 to 8 weeks of the end of the treatment. These patients had received the drug for periods ranging from 6 to 10 days. In 3 of them the relapses were mild, but in the other 3 the symptoms were fairly severe. In all of these patients the infection was adequately controlled by a further course of treatment. Symptoms and signs of a drug-induced exacerbation developed in 5 patients during the first or second day of treatment, but in no case did this cause alarm. No other serious toxic effects were encountered.

The uniform manner in which all patients improved after treatment was started, even though cure was not established, was a striking feature and was thought to indicate a beneficial therapeutic action. The authors considered that the high relapse rate was due to the short period of treatment and that a more prolonged course (even up to 4 to 6 weeks) might be justified. For the average adult the suggested optimum dosage is about 6 g. per day. A comparison between the temperature charts of 11 patients treated with aureomycin and the temperature charts of those in the present series given chloramphenicol does not reveal any great difference in the efficacy of the two drugs.

T. Anderson.

Lymph-node Culture; a Rapid and Accurate Method for the Bacteriological Diagnosis of Acute Brucellosis. JANBON, M., BERTRAND, L., and QUATREFAGES, H. (1950). *Pr. méd.*, **58**, 1355.

In view of the frequent enlargement of the lymph nodes in brucellosis, the authors suggest the removal of a lymph node under strictly aseptic conditions for rapid bacteriological examination as a means of diagnosis. The tissue is ground up and cultivated on "bacto" tryptose agar. After 3 days of incubation the growth of small colonies is visible; these colonies are translucent and have a granular appearance and are identified by direct examination and agglutination. Any culture developing before the third day is due to contamination. Of 53 examinations in 50 cases of brucellosis, 33 (60%) showed a positive result, 12 were negative, and 8 were contaminated. In 18 comparative tests in cases of other diseases the investigations were negative.

Franz Heimann.

Studies of Terramycin, *in vivo* and *in vitro*. BLISS, E. A., WARTH, P. T., CHANDLER, C. A., and LONG, P. H. (1950). *Bull. Johns Hopk. Hosp.*, **87**, 171.

A series of *in vivo* and *in vitro* tests upon terramycin are reported, and its general physical properties are noted.

The minimal inhibitory concentrations of terramycin were about $\frac{1}{8}$ to $\frac{2}{8}$ those of aureomycin against several strains of 12 different genera, except in the case of *Pseudomonas aeruginosa*, in which they were 1/10 to 1/20. In the same way the rate of deterioration due to prolonged incubation and changes in *p*H and temperature was studied. Filtration through a Seitz filter pad was shown to have no effect on activity.

A concentration of 20 μg. per ml. sterilized a standard culture of *Bacterium coli* after a time, but if concentrations up to 10 μg. per ml. were used a slow initial decline in numbers was always followed by growth. Organisms surviving after 4 days in 5 μg. terramycin per ml. had not become resistant, a decline in activity of the antibiotic over this period accounting for their growth. This decline was not greater in contact with *Bacterium coli* than in a control non-inoculated broth.

Serum levels and urinary excretion were studied in 2 normal subjects after a dose of 2 g. terramycin hydrochloride by mouth. The highest serum concentration (4 to 5 μg. per ml.) occurred at 2 to 4 hours, and maximal excretion at 4 hours, after ingestion.

Terramycin was more effective than aureomycin in protecting mice against *Klebsiella pneumoniae* and *Haemophilus influenzae*, but less protective against the haemolytic streptococci and the pneumococcus.

E. A. Brown.

Modern Therapy of Plague. MEYER, K. F. (1950). *J. Amer. med. Ass.*, **144**, 982.

On the basis of impressive experiences in the laboratory and in field trials, the following modern therapy is recommended in cases in which the presence of plague is suspected.

Streptomycin should be given in large doses (4 g.) at first, but for reasons of economy the dose may be reduced safely on the third and fourth day of recovery. After the fifth day, streptomycin may be replaced by sulphadiazine or sulphamerazine (4 g. daily). In severe septicaemia, and particularly in pneumonic plague, the initial daily dose of 4 g. of streptomycin should be supplemented by oral administration of aureomycin, chloramphenicol, or terramycin (2 to 4 g.) and anti-plague immune serum globulin (rabbit) available at the National Institutes of Health. If the patient does not respond to streptomycin and sulphonamide treatment in 2 or 3 days, even when optimal and large initial doses have been given, the infecting strain may be resistant to these antibiotics. In such cases chloramphenicol, aureomycin, terramycin, aerosporin, or neomycin may prove beneficial.

When antibiotics are not available, sulphadiazine or sulphamerazine in an initial dose of 4 g., followed by 1 g. every 4 hours for not more than 10 days, has proved highly effective in the treatment of uncomplicated bubonic plague.

Contacts exposed to secondary or primary pneumonic plague should be given 2 or 3 g. of sulphadiazine or sulphamerazine daily for 5 days. With the onset of symptoms, intensive treatment with antibiotics must be instituted.—[Author's summary.]

Preliminary Report on 4:4′-Diaminodiphenyl Sulfone (DDS) Treatment of Leprosy. MUIR, E. (1950). *Int. J. Leprosy*, **18**, 299.

4 : 4′-Diaminodiphenyl sulphone (DDS) is the parent substance of the proprietary sulphones and has hitherto been regarded as too toxic for human use. The drug was given orally in a 2·5% suspension in gum-acacia in ordinary uncomplicated cases of leprosy in doses of 4 mg. per kg. body weight, the average dose being 0·2 g. Intolerance was indicated by anaemia, and necessitated a smaller dose; a small dose was also given to patients who had complications or were very ill, and to those with lepra reactions. In all, 94 cases were treated, divided into three groups: (1) advanced lepromatous cases with complications such as lepromatous ulcers, blocked nose, eye conditions, and lepra reaction, in which the earlier effects could be suitably tested; (2) uncomplicated cases in good health; (3) tuberculoid cases with widespread lesions (5 cases). Clinical and bacteriological improvement was assessed, the latter by study of smears from five suitable skin points by the scraped incision method. Definite clinical improvement was obtained in 48, and considerable improvement in 24, of the 50 cases treated over 9 months; bacteriological improvement occurred in 10 of the same series of cases. Sulphone reactions were seen, similar to lepra reactions except that the former

were followed by improvement. Drug fastness was not encountered. It is concluded that DDS is effective, and, with proper precautions, safe; it has the great advantage of cheapness. *J. L. Markson.*

An Acid-fast Microorganism Cultivated from Leprous Material. Bacteriological and Serological Observations. TERNI, M. (1950). *Int. J. Leprosy*, **18**, 161.

An acid-fast organism has been obtained in culture during the course of numerous attempts to isolate leprosy bacilli in the media of Dubos and Petragnani and in the larvae of *Galleria mellonella*. Material from a skin leproma was inoculated into six larvae, and 2 days later a transfer was made from one larva into tubes of medium. A single colony was obtained in a tube of Petragnani's medium after incubation for 3 months. In an investigation of the characteristics of the organism it was found to be acid-fast, Gram-positive, aerobic, and non-motile; it grew best at 37° C., and required glycerol. It was not pathogenic for laboratory animals. Antigens prepared from cultures of the organism reacted in complement-fixation tests with sera of leprous patients up to titres of 1 in 320. The organisms were not lysed by bacteriophages active against five species of mycobacteria, and it is concluded from a review of their properties that they were neither tubercle bacilli nor saprophytic contaminants. *D. G. Bauer.*

BIOCHEMISTRY

Iron Metabolism. The Pathophysiology of Iron Storage. FINCH, C. A., HEGSTED, M., KINNEY, T. D., THOMAS, E. D., RATH, C. E., HASKINS, D., FINCH, S., and FLUHARTY, R. G. (1950). *Blood*, **5**, 983.

This paper describes experiments carried out on animals to elucidate questions of iron storage and utilization. The distribution and availability of stored iron were studied in dogs, the degree of localization of stored iron in rats, and changes in total body content of iron under various conditions in mice. In addition the organs of patients with haemochromatosis were analysed post mortem for iron content.

Storage iron—the iron which can be mobilized from the body tissues for the formation of haemoglobin—is present intracellularly in the form of two similar compounds, ferritin and haemosiderin. The body of a normal man contains about 5,000 mg. of iron, of which storage iron constitutes 20%; since only a fraction of a milligram is excreted daily in the urine and faeces, excess iron must be stored and is found in the reticulo-endothelial or parenchymal cells, the liver being the most important organ involved. In iron deficiency anaemia the absorption of iron is increased, but the iron is used directly for haemoglobin synthesis and is not stored. The iron stores thus remain depleted for a period which may extend over many months.

The authors state that increased absorption and storage of iron may be produced in animals by altering the composition of the diet. In such cases there is an increase in the serum and liver iron content, and eventually haemosiderosis develops. The distribution of iron is similar to that seen in cases of haemochromatosis. In animals given intravenous injections of iron-ascorbate gelatin and observed for periods up to 2 years massive deposits of iron were found in the reticulo-endothelial system, whereas excess iron given by mouth or injected in small quantities in the form of soluble salts was stored predominantly in the liver. When erythrocytes labelled with radioactive iron were injected into rats, it was found that the spleen absorbed most of the erythrocytes, whereas haemoglobin injected in solution was mostly concentrated in the kidneys. Iron localization after the breakdown of blood thus depends on whether the breakdown is intravascular or extravascular. Focal deposits of iron in various organs such as the brain, lungs, and skin indicate previous breakdown of blood in the areas involved. The serum iron level, although always low in the presence of infection, is a

measure of excess iron storage, while the amount of stored iron may also be determined by examination of the liver or bone marrow. No evidence was found that the stored iron in haemochromatosis differs from that stored when iron is injected. In dietary or idiopathic haemochromatosis there is an excess absorption of iron, and it is stated that by giving large doses of iron and a diet of low phosphate content an increase in body iron storage may be produced.

T. M. Pollock.

Electrolyte Studies on Patients with Cirrhosis of the Liver. EISENMENGER, W. J., BLONDHELM, S. H., BONGIOVANNI, A. M., and KUNKEL, H. G. (1950). *J. clin. Invest.*, **29**, 1491.

Exhaustive balance studies were carried out on 41 patients suffering from cirrhosis of the liver. In 18 patients there was marked ascites and in 10 moderate ascites; in the remaining 13 there was no ascites. The patients with marked ascites had Laennec's cirrhosis, and among the others were some cases of biliary or " postnecrotic " cirrhosis. Sodium and potassium content of the patient's food, serum, urine, sweat, and faeces were estimated by a flame photometer. The food and faeces were ashed before analysis. Since some urines contained more than three hundred times as much potassium as sodium the question whether such large amounts of potassium interfered with the sodium estimations was studied, but the error was found to be small. Chloride concentration was also estimated in the food, serum, urine, and ascitic fluid, and nitrogen balance was estimated in some of the patients.

It was found that patients with severe ascites excreted extremely small amounts of sodium (their urinary excretion was usually below 1 mEq. daily and never above 4 mEq.), and excretion remained low even on a high intake of sodium. Those with ascites excreted by various means all the sodium they ate. Those with moderate ascites generally excreted less sodium in their urine than those who had no ascites [and it must be concluded that they retained some of the sodium they ate]. Serum sodium levels were low in patients with ascites (about 140 mEq. per 100 ml.), and low normal in the others. After paracentesis the serum sodium level fell even lower than before. If the intake of sodium was reduced to between 15 and 17 mEq. daily, the retention of fluid immediately stopped in those patients who were suffering from ascites. At the same time, however, potassium and nitrogen were retained in the body. Conversely, the serum sodium level increased and more sodium was excreted when patients with ascites began to improve, even before water balance could show that less water was being retained in the body. Since the saliva and sweat of patients suffering from ascites also contained less sodium than normal, the authors suggest that an overaction of the adrenal cortex may play a part in the retention of sodium in cirrhosis of the liver. This contention is supported by reference to some data which are to be published later.

The Thigh-Neck Clearance: a Simplified Radioactive Test of Thyroid Function. FOOTE, J. B., and MACLAGAN, N. F. (1951). *Lancet*, **1**, 868.

This test is based on measurements of radioactivity made at fixed distances (11 cm.) from the neck and thigh. A dose of 30 μc. of radioactive iodine (^{131}I) is administered orally and serial measurements are made over neck and thigh until the maximum thigh count has been reached. Measurements over the neck are made for a duration of 1 minute, and those over the thigh for 10 minutes. The " thigh-neck clearance " is determined at the time of the maximum thigh count, and is defined as the rate of uptake in the neck expressed as the increase of neck counts per hour divided by the thigh count. A series of 100 persons was studied by this test. In 24 normal subjects the thigh-neck clearance ranged from 1 to 9, and in 14 patients with definite thyrotoxicosis the values ranged from 21 to 108. A simple qualitative test for urinary iodide is also described. This proved useful in detecting patients who had received treatment with iodine containing

substances which would invalidate the radioactive test. *G. Ansell.*

HAEMATOLOGY

The One-stage Prothrombin Consumption Test. Clinical Value in the Identification of Thromboplastin-deficiency Diseases. STEFANINI, M., and CROSBY, W. H. (1950). *Blood*, **5**, 964.

The formation of a solid clot does not represent the end-point of the intricate complex of reactions leading to the coagulation of blood or plasma. In haemophilia, acquired haemophilia-like diseases, and severe thrombocytopenia there is always impaired activation by thromboplastin. In the presence of a minimal amount of thromboplastin enough thrombin may, however, be formed eventually to clot fibrinogen, but much residual prothrombin will be found in the serum. The purpose of the prothrombin consumption test is to identify this residual prothrombin activity. The test requires deprothrombinized plasma, thromboplastin, and a standard curve of plasma prothrombin activity. This curve is prepared by determining the prothrombin time of serial dilutions of deprothrombinized with normal plasma. Each mixture is assumed to possess a percentage of normal prothrombin activity corresponding to the percentage of normal plasma it contains, and the correlation of prothrombin time with this figure results in an exponential curve. Reference to this curve allows the conversion of other prothrombin time values into percentages of normal prothrombin activity. For the test, the serum from 2 ml. of clotted blood is mixed with $\frac{1}{10}$ volume of sodium citrate and incubated for 30 minutes to allow the neutralization of thrombin. The clotting time is then recorded after adding, in rapid succession to a test tube kept at 37° C., 0.1 ml. of each of the following: (1) deprothrombinized plasma; (2) 0.02 M calcium chloride; (3) thromboplastin; and (4) the serum to be tested.

The serum prothrombin activity was below 10% in $\frac{1}{10}$ normal subjects. In 2 cases of thrombocytopenic purpura the values were 87% and 63% respectively; 24 hours after splenectomy they had fallen to 6% and 0.8% respectively. Out of 7 cases of thrombocytopenia due to leukaemia in only one was the value below 10% (9.8%); in one it was 15%, in 2 between 30% and 40%, and in the remaining 3 above 60%. Similar results were obtained in one case of aplastic anaemia (34%) and 2 cases of haemophilia (68% and 91%); in 2 cases of pseudohaemophilia, 5 cases of hypothrombinaemia due to dicoumarol treatment, and 3 cases of polycythemia vera the values obtained were all below 10%.

The authors have examined the effect on the validity of the test of the presence of different amounts of serum accelerator and conclude that it is unaffected. Though normal serum has a high accelerator content, there is little prothrombin to act upon. Even 100% acceleration of prothrombin activity is of no practical significance when the residual level is only 2%. On the other hand, in a serum where there has been little prothrombin consumption, accelerator content is negligible. *H. Lehmann.*

Serum Prothrombin Time, a Composite Effect. An Analysis of the Factors Involved. STEFANINI, M., and CROSNY, W. H. (1950). *Amer. J. clin. Path.*, **20**, 1026.

Quick's one-stage prothrombin consumption test (Quick, Shanberge, and Stefanini, *J. Lab. clin. Med.*, 1949, **34**, 761) determines the prothrombin content of serum and is valuable for the diagnosis of haemophilia and thrombocytopenic purpura. This paper is a study of the influence different variables exert on this procedure.

There are at least five coagulation factors involved: (1) the concentration of unconverted prothrombin in the serum; (2) the thrombin content of the serum, that is, that proportion of thrombin which has not been absorbed by fibrin or neutralized by the natural antithrombin; (3) the "accelerator effect" of the serum; (4) the labile factor "V," plasma Ac-globulin not activated during coagulation; (5) (if coagulation has been completed more than 2 hours before the serum is tested) activation of a postulated

prothrombin precursor. Of these five factors, three can be disregarded: the thrombin can be neutralized by incubation of the serum in the presence of citrate for 15 minutes, the concentration of labile factor can be ignored, as it is added in excess, and the prothrombin precursor will not be activated provided not more than 2 hours are allowed to pass between coagulation of the plasma and the testing of the serum. The test is therefore mainly influenced by the concentration of residual prothrombin and the accelerator effect of the serum. Thus the test reflects not only prothrombin concentration but also the velocity of the conversion of prothrombin into thrombin; it is a measurement of serum prothrombin activity rather than of concentration. However, in normal sera where there is drastic depletion of prothrombin no accelerator effect, however powerful, can compensate for this consumption; and since in abnormal sera in which consumption of prothrombin is defective there is only a weak accelerator effect, from the practical point of view Quick's test remains a measure of prothrombin concentration. *H. Lehmann.*

The Effect of Choline on Haematopoiesis. CANIGGIA, A. (1950). *Haematologica*, **34**, 625.

The effect of choline on the blood picture was studied in 12 patients—2 with pernicious anaemia, 2 with haemolytic jaundice, 1 with post-haemorrhagic anaemia, 1 with severe hypochromic anaemia of unknown origin, and 6 with post-infective anaemia. In all cases peripheral blood and bone-marrow smears were examined. Two patients were treated with choline chloride 1 g. a day for 12 to 20 days, 8 with 2 g. a day for 10 to 25 days, and 2 with 2 g. a day for 15 days plus 0.6 g. of iron a day as sulphate. In all cases the regimen of choline supplements produced marked changes in the blood and marrow. Erythrocytes increased in number, with a moderate reticulocytosis, diminution in mean corpuscular volume, restoration of haemoglobin content, and there was a diminution in the number of megaloblasts and accelerated maturation of erythroblasts. Moderate leucocytosis and shift of Arneth's curve to the left was noted. The addition of iron increased the haemoglobin level and prevented the fall in mean corpuscular volume.

It is suggested that choline has a threefold action: mobilizing and reducing hepatic fat; playing a part in the synthesis of methionine; and acting directly on the bone marrow as a stimulant to cellular proliferation. Choline should be included in anti-anaemic regimens. [There is no mention in this paper of control observations on similar patients.] *James D. P. Graham.*

MORBID ANATOMY AND HISTOLOGY

Prognosis in Cutaneous and Ocular Malignant Melanoma: A Study of 222 Cases. WRIGHT, C. J. E. (1949). *J. Path. Bact.*, **61**, 507.

A combined study of pathology and survival in cases of malignant melanoma was made. There were 109 cases of malignant skin or mucosal tumour, 27 of doubtful malignancy, and 89 malignant ocular tumours. The 5-year survival rate was 28.6% and the 10-year rate 12.5% in the first group, whereas in the cases of eye tumour the prognosis was much better (62.5% and 39.4% respectively). With tumours of doubtful malignancy (mostly in children and young adults) survival was almost indefinite. In a few cases the value of block dissection of regional lymph nodes was shown, even when they were not apparently enlarged, and in one case there was a long survival period after the microscopical finding of melanoma in a lymph node. In the elderly and with large tumours the prognosis generally is not good. For cases of doubtful malignancy of course only local excision is required. It is finally concluded that if a patient with malignant melanoma of the skin survives 5 years after its excision without local recurrence or lymph-node involvement, there is a good chance of his remaining free of growth; the corresponding period for eye tumours is 10 years. *W. S. Killpack.*

Squamous Metaplasia of the Peritoneum. CROME, L. (1950). *J. Path. Bact.*, **62**, 61.

The author first reviews the scanty literature on squamous metaplasia of the peritoneum and then describes 4 specimens of his own, removed surgically at St. Mary's Hospital, London, and coming from 2 cases of strangulated intestine, one of acute appendicitis, and one of carcinoma of the uterus in which panhysterectomy had been performed. The peritoneum in these cases showed tiny nodules, up to 4 cm. in diameter, of squamous epithelium together with little islands of epithelium under the surface and various stages in the conversion of these islands into cysts lined by a single layer of flattened cells. The author suggests that groups of serosal cells undergo metaplasia to squamous epithelium and that this epithelium becomes buried during the organization of fibrin deposited on the peritoneal surface. He considers that endometriosis is also due to serosal metaplasia and regards these findings as providing further indirect evidence to support his views on its origin. The author points out that, if the occurrence of peritoneal metaplasia be established, it becomes possible to view more favourably the descriptions in the literature of various types of primary epithelial tumour arising in the peritoneum.

Peter Harvey.

Smooth-Muscle Tumours of the Gastro-Intestinal Tract. COWDELL, R. H. (1950). *Brit. J. Surg.*, **38**, 3.

Of 370 stomach tumours examined, 9 were leiomyomata or leiomyosarcomata.

These tumours may be intragastric, intramucosal, or extragastric. Histologically there is a variation in pattern in different parts of the same tumour, and it is often difficult to decide whether a tumour is benign or malignant, the frequency of mitoses being the most valuable single criterion of malignancy.

The most common symptom produced by these tumours is haemorrhage, either into the gastro-intestinal tract or into the peritoneal cavity. Ulceration of a gastric leiomyoma produces symptoms of chronic peptic ulceration. Occasionally a patient may complain of an intra-abdominal mass.

Ependymal Tumours of the Spinal Cord and Filum Terminale. WERTHEIMER, P., ALLÈGRE, G., and GARDE, A. (1950). *Rev. neurol.*, **82**, 153.

Seven cases of ependymal tumour of the spinal cord, including the filum terminale, are briefly described by the authors; histological examination had previously led to the diagnosis of neuroepithelioma, but renewed study revealed the true nature of these lesions. The authors distinguish between ependymoma and ependymoglioma. These ependymal tumours are relatively benign and can occasionally be removed *in toto*.

Theca-cell Tumors. SPARLING, D. W. (1950). *Amer. J. Obstet. Gynec.*, **59**, 1279.

The author presents individual reports on a series of 11 cases of theca-cell tumour.

The growths varied in size from a firm, rubbery nodule in the wall of a cyst to that of a grapefruit. They were all unilateral and their only distinguishing feature was their yellow or yellowish-orange colour. The endometrial histology was determined in 6 cases, and in 3 of these showed a "Swiss-cheese" pattern of hyperoestrinism; this was probably due to the presence of cells of the granulosa type. The histological differentiation of these tumours from granulosa-cell tumours may be difficult, but is helped by staining with Sudan III, which shows the fat to be intracellular in theca-cell tumours and extracellular in granulosa-cell tumours; moreover, the cells of the thecal tumour are surrounded by a fine connective-tissue reticulum, whereas the granulosa cells are not so enclosed, or only in groups.

The author considers these growths to be benign, although in one patient the tumour was associated with an adenocarcinoma of the fundus uteri.

The Morbid Anatomy of Tuberculosis in Old Age. ROULET, F. (1950). *Ann. Méd.*, **51**, 69.

In 270 necropsies carried out at the University of Basle on subjects over 60 years old, frank evidence of tuberculosis was present, although it was the cause of death in only 90 cases. The active focus was found in 245 cases around an old calcified or ossified nodule in the tracheo-bronchial lymph nodes; The lungs in these cases revealed no active primary focus, though an old, calcified, inactive lesion was often discovered. It is argued that active tuberculosis in old age is frequently caused by reactivation of an old lesion in the lymph nodes around the bifurcation of the trachea, possibly due to the chronic inflammation of the lower trachea and main bronchi, which is extremely common in old people. Tuberculosis of the tracheo-bronchial nodes frequently leads to complications: of the 245 cases of tracheo-bronchial-node infection, there was also frank tuberculous infection of the lungs in 35: in 12 cases there was a generalized haematogenous spread either from a focus in the tracheo-bronchial nodes or from an alternative site of active infection; in 13 cases there were foci both in the tracheo-bronchial nodes and elsewhere, but no generalized spread; and in 25 cases haematogenous spread had occurred with tracheo-bronchial nodes as the only possible primary source. In the remaining 160 cases the infection was confined to the tracheo-bronchial nodes.

Speaking generally, miliary tuberculosis develops more rapidly in the old than at other ages. The appearances are classical, though the tubercles may be rather large—as big as a pea or a hazel nut. In the whole series there were 16 cases of miliary spread amongst the 43 cases of frank pulmonary tuberculosis. Tuberculosis of the serous cavities is not uncommon in the elderly. Pulmonary tuberculosis may be of the chronic apical fibro-caseous type with cavities, or of the ulcerative caseous type of acute tuberculosis, the frequent occurrence of which in old people may possibly be due to reduced general resistance and antibody response.

Glomerulonephrosis. A Morphologic Manifestation of Renal Cortical Ischemia in Toxic Oliguria and Lower Nephron Nephrosis. FRENCH, A. J. (1950). *Arch. Path.*, **49**, 43.

The histopathological findings in the kidneys of 20 specially selected cases of glomerulonephrosis are described, special stress being laid on the glomerular changes. This study was undertaken in the hope of explaining the anuria or oliguria which accompanies lower nephron nephrosis (" tubular " or " toxic " nephritis), in which condition, on physiological grounds, there should be polyuria if a tubular lesion alone be present. For this purpose it was essential to eliminate cases of primary or secondary renal disease, eclampsia, and cardiovascular hypertension.

The changes found in the glomeruli, tubules, and medulla in sections of the kidneys (mostly stained with haematoxylin and eosin) are tabulated. Glomerular ischaemia, together with thickening of the capillary walls, was found in every case. The presence of granular material staining grey-blue in the glomerular spaces and proximal tubules was noted, this being regarded as the precursor of the protein casts seen in the distal convoluted tubules in all except one of these cases. *R. B. T. Baldwin.*

Corrections.—Dr. Varadi writes: I shall be obliged if you will print the following corrections to my article " Combined Fragility Test for Congenital Haemolytic Jaundice " (*J. clin. Path.*, **4**, 221). On page 222 in paragraph 3 read 0.021% instead of 0.21% NaCl, and, on page 224 paragraph 2 of the Appendix, marrow puncture instead of culture.

We much regret that in the description of a cell-suspension mixer by Dr. Matthews (*J. clin. Path.*, **4**, 383) the pipettes were described as 20 ml. and 50 ml. These should be 20 c.mm. and 50 c.mm.

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